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SEX DIFFERENCE IN THE ENDOCRINE RESPONSE OF ADULT DOMESTIC CHICKENS TO GONADOTROPHIN-RELEASING HORMONE-I

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February 1993

DECLARATION

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree elsewhere. The work presented herein is my own, and all assistance given to me is acknowledged.

Raymond Chee Ming LIU

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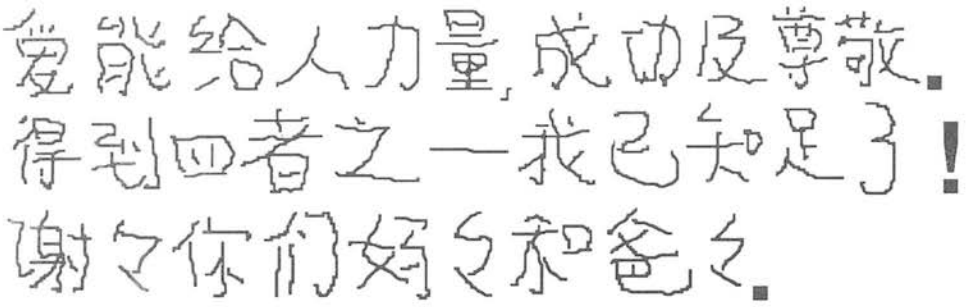

[Through love comes strength comes success comes respect.....one out of four ain't bad!]
For their love and encouragement, I thank my parents.

Ladies and gentlemen of whom I have extolled, I salute you!

PUBLICATIONS ARISING FROM THIS THESIS

ABSTRACTS

A sex difference in responsiveness to gonadotrophin-releasing hormone in chicken pituitary cells: a study on intracellular calcium. Liu RCM & Lea RW (1990) ***Regulatory Peptides***. 30: Abstract 48

An oestradiol-dependent component of the sex difference in the LH response of adult chickens to GnRH-I. Liu RCM, Main GM, Lea RW & Sharp PJ (1991) ***Journal of Reproduction and Fertility.*** Abstract Series 7, no. 61

Gonadal steroids directly modify the LH response to GnRH-I in cultured anterior pituitary cells from juvenile chickens. Liu RCM, Sharp PJ & Lea RW (1991) ***Journal of Reproduction and Fertility.*** Abstract Series 8, no. 96

Testosterone inhibits the function of immature chicken gonadotroph cells by aromatase-dependent and independent mechanisms. Liu RCM, Sharp PJ & Lea RW (1992) ***Fifth International Symposium on Avian Endocrinology Programme and Abstracts***, AFRC Institute of Animal Physiology and Genetics Research, Edinburgh. ISBN 0 7084 05185

PAPERS SUBMITTED FOR PUBLICATION

Sex differences in the hormones of the hypothalamic-gonadotroph axis of adult domestic chickens. ***Journal of Endocrinology.***

Effect of age of donor on culture conditions required to maintain responsiveness of dispersed chicken anterior pituitary cells to GnRH-I. ***British Poultry Science.***

Intracellular mechanisms involved in the sex difference in the LH response of the adult domestic chicken to GnRH-I. ***General and Comparative Endocrinology.***

Ultrastructural studies on lipid-containing cells of the anterior pituitary gland of the domestic chicken (*Gallus domesticus*). ***The Anatomical Record.***

ABBREVIATIONS

5α-DHT	5α-dihydrotestosterone
5ß-DHT	5ß-dihydrotestosterone
AC	acid phosphatase
AUC	area-under-the-curve
BSA	bovine serum albumin
cAMP	cyclic 3'5'-adenosine monophosphate
CM	cell membrane
ΔLH	incremental change in LH concentration
DARS	donkey anti-rabbit serum
dbcAMP	dibutyryl cyclic 3'5'-adenosine monophosphate
DG	1,2-diacylglycerol
DMEM	Dulbecco's modified Eagles medium
EBSS	Earles balanced salt solution
ECD	equivalent cell diameter
ED_{50}	dose required for half-maximum stimulation
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N',N',N',N'-tetra acetic acid
FCS	foetal calf serum
FSH	follicle-stimulating hormone
G-prot	guanosine nucleotide binding protein
GC	Golgi complex
GFA	granule fractional area
GnRH	gonadotrophin-releasing hormone
GnRH-I	chicken (Gln^8)-gonadotrophin-releasing hormone
GnRH-II	chicken (His^5-Trp^7-Tyr^8)-gonadotrophin-releasing hormone
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
IC_{50}	dose required for half-maximum inhibition
IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,4,5,6-tetrakisphosphate
IP_6	inositol 1,2,3,4,5,6-hexakisphosphate
LH	luteinizing hormone
Ld	lipid
Lys	possible lysosome
M199	Medium 199
MBH	mediobasal hypothalamus
M	mitochondrion
mRNA	messenger ribonucleic acid
N	nucleus
NBCS	newborn calf serum
NFP	nifedipine
NRS	normal rabbit serum
NSB	non-specific binding
NSS	normal sheep serum
OB	oestradiol benzoate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
Pn	perinuclear
POA	preoptic area
RIA	radioimmunoassay
Rib	ribosomes
RRP	'readily releaseable' pool
SER	smooth endoplasmic reticulum
Sp	subplasmalemmal
TMA-DPH	1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene
TPA	12-O-tetradecanoyl 13-phorbol acetate
TRIS	Tris[hydroxymethyl]amino-methane
TSH	thyroid-stimulating hormone
V	veratridine
VSCC	voltage-sensitive Ca^{2+} channel
w/v	weight per volume

ABSTRACT

The responsiveness and sensitivity to an injection of chicken [Gln^8]-gonadotrophin-releasing hormone (GnRH-I) is lower, and the duration of increased plasma luteinizing hormone (LH) is more prolonged, in laying hens than in adult cockerels (*Gallus domesticus*). This thesis sought to further characterise and establish an oestrogen-dependency of these sex differences *in vivo* and *in vitro*, and to determine the mechanism for sexual differentiation of GnRH-I-induced LH secretion.

The magnitude and profile of the GnRH-I-stimulated LH response, and the sensitivity to GnRH-I were shown to be functions of the anterior pituitary gland. There was less LH in gonadotroph cells from laying hens than from adult cockerels, but no sex difference between juveniles. The low responsiveness to GnRH-I of laying hens was reproduced by oestrogen treatment of adult cockerels *in vivo* and pituitary cells from juvenile chickens *in vitro*, through depression of the total and readily releaseable pool (RRP) of pituitary LH. The secretion of LH from GnRH-I-stimulated isolated pituitary glands was biphasic, consisting of a spike and a plateau. Sexual differentiation of the magnitude of the spike of LH release was determined by the smaller RRP of LH, and the absence of an extracellular Ca^{2+}-independent and an L-type-Ca^{2+} channel-dependent component of LH secretion from pituitary glands from laying hens, compared with that from adult cockerels. A decrease in spike-to-plateau phase ratio of GnRH-I-induced LH secretion was observed *in vitro* in pituitary glands from hens at onset of sexual maturity. This decrease in ratio was simulated in pituitary glands from adult cockerels *in vitro* after incubation with 17ß-oestradiol. The membrane fluidity of pituitary cells from laying hens was lower than that from adult cockerels and shown to be induced in pituitary cells from juvenile chickens by treatment with 17ß-oestradiol. Attempts to establish an oestrogen-dependency of the sexually differentiated sensitivity of adult chickens to GnRH-I, or to relate it to a sex difference in pituitary GnRH-receptors were unsuccessful. The sex difference in GnRH-I-induced duration of increased plasma LH could not be reproduced *in vitro* and therefore depends on an extrapituitary mechanism.

These sex differences became fully established after sexual maturation, with similar GnRH-I-induced LH responses being found in adult cockerels and juveniles of both sex. It is therefore concluded that sexual differentiation of these LH responses results from the maturational increase in plasma 17ß-oestradiol in hens.

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1.1 General

The evolution of the reproductive system ensures that young are born at a time of year when the availability of food is optimal for their survival. In order to anticipate seasonal availability of food, many animals use seasonal changes in daylength to initiate the onset and termination of reproductive activity. The fact that seasonal changes in daylength are used in this way implies that this environmental information is transduced via sensory inputs to the central nervous system into hormonal signals which induce seasonal gonadal development.

Within the central nervous system a part of the diencephalon, the hypothalamus, is the principal site where environmental information in the form of neural inputs is transduced into a neuroendocrine output. The neurosecretion is a decapeptide, gonadotrophin-releasing hormone (GnRH). It is secreted from nerve terminals on the surface of the hypothalamus in a region known as the median eminence, into a portal vascular system which drains directly into the anterior pituitary gland. There GnRH stimulates the synthesis and release of two glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) collectively termed the gonadotrophins.

The gonadotrophins are secreted into the peripheral circulation to stimulate the development of the gonads and the secretion of gonadal hormones. The small amount of GnRH released from the hypothalamus would itself be insufficient to stimulate gonadal function directly. The function of the anterior pituitary gland is therefore to amplify a small neuroendocrine signal, GnRH release, into a larger endocrine output of gonadotrophins which are secreted in sufficient amounts into the peripheral circulation to stimulate gonadal function. The gonads secrete sex steroids, principally testosterone in the male, and 17ß-oestradiol and progesterone in the female. These steroids stimulate sexual behaviour and the development of secondary sexual characteristics.

The hypothalamus, anterior pituitary gland and the gonads form the hypothalamic-pituitary-gonadal axis (HPGA). As described so far, the HPGA is 'open-ended' with no mechanism to ensure homeostasis of hormonal output. This is achieved by the negative feedback actions of gonadal steroids which depress the secretion of the gonadotrophins by acting directly on the anterior pituitary gland or by suppressing the secretion of GnRH by acting on the hypothalamus (FIGURE 1.1). This negative feedback mechanism maintains

the 'tonic' secretion of gonadotrophins at plasma concentrations sufficient to maintain gonadal activity. Outside the breeding season the hypothalamic 'set point' of the whole system is lowered to reduce the secretion of GnRH independently of steroid inhibitory feedback.

This thesis is concerned with an aspect of the tonic secretion of LH in the domestic chicken. In this species, there is a sex difference in the tonic secretion of LH which is lower in the adult female than in the adult male (Sterling & Sharp, 1984; Sharp *et al.*, 1987). This is known to be due to the LH response of the anterior pituitary gland to GnRH being less in the female than in the male (Sterling & Sharp, 1984; Sharp *et al.*, 1987). The role of gonadal steroids in this sexually differentiated response to GnRH is explored in this thesis.

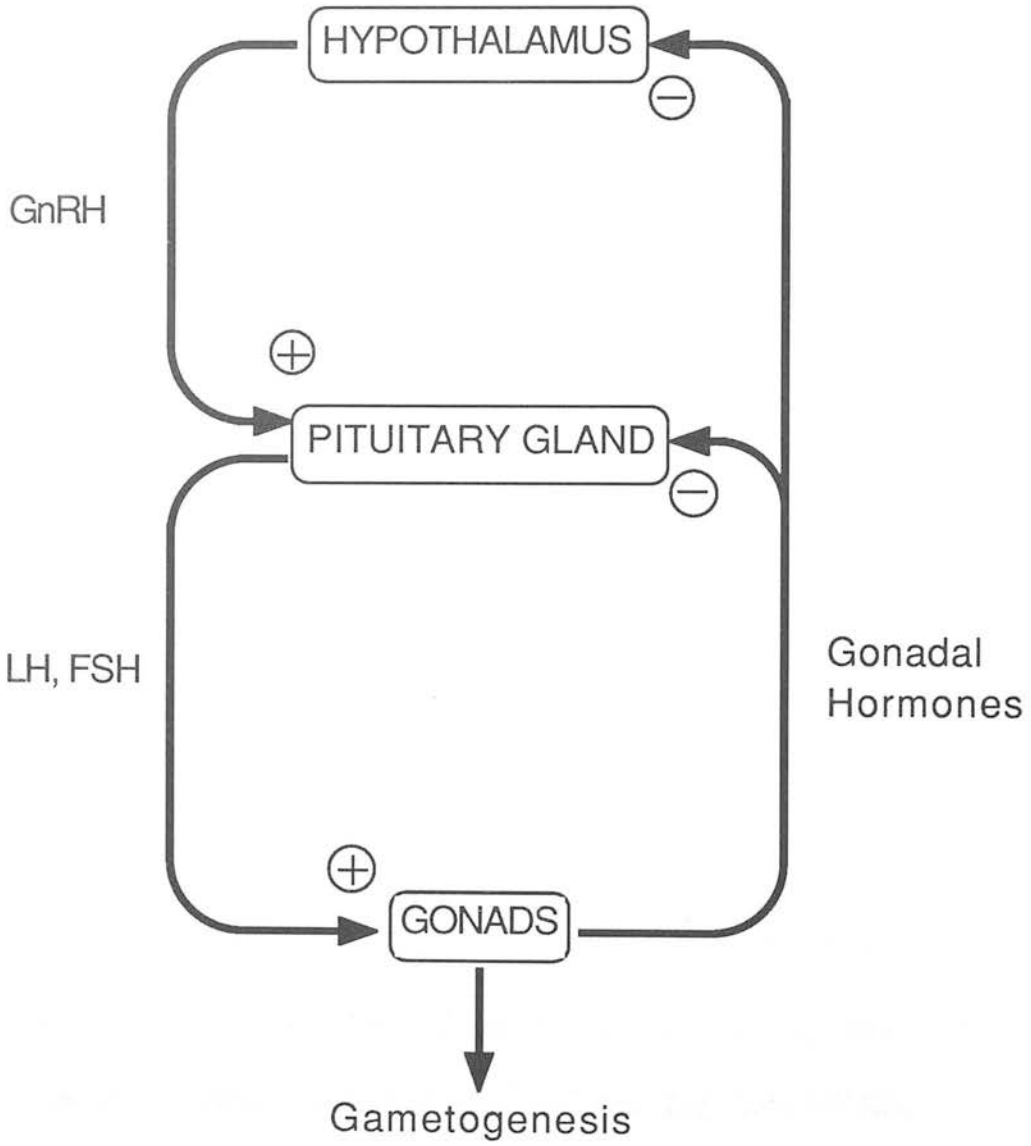


FIGURE 1.1: Basic principles of the feedback relationships of the hypothalamic-pituitary-gonadal axis in the control of gametogenesis.

1.2 Historical perspective

The importance of the central nervous system in the control of gonadal function in birds was first demonstrated by lesioning studies in the duck (Benoit & Assenmacher, 1955, 1959). Later studies in the domestic chicken also showed the association between the activity of the hypothalamus and gonadotrophin secretion. Thus destruction of the preoptic area of the hypothalamus reduces gonadotrophin secretion and causes regression of the gonads (Shirley & Nalbandov, 1956; Ralph, 1959; Ralph & Fraps, 1959a, 1959b; Egge & Chiasson, 1963; Ravona *et al.*, 1973). Furthermore in quail, electrical stimulation of the hypothalamus increases gonadotrophin secretion (Davies & Follett, 1975a, 1980), and lesions of the preoptic area block photoperiodically induced release of gonadotrophins and testicular growth (Sharp & Follett, 1969b; Davies & Follett, 1975b, 1980; Oliver *et al.*, 1979).

The observation that the avian hypothalamus contains a substance capable of stimulating LH release from anterior pituitary tissue *in vitro* (Follett, 1970; Smith & Follett, 1972), and of inducing premature ovulation in laying hens (Clark & Fraps, 1967; Opel & Lepore, 1967, 1972) provided early evidence for an 'ovulation-hormone releasing factor'. The isolation and characterisation of a gonadotrophin-releasing peptide extracted from sheep hypothalamic tissue (FIGURE 1.2) was followed by the demonstration of gonadotrophin-releasing activity in many species including the chicken (reviewed by Schally *et al.*, 1972; van Tienhoven & Schally, 1972). Mammalian gonadotrophin-releasing hormone (mGnRH) releases LH and FSH from the chicken and quail *in vivo* (Furr *et al.*, 1973b; Reeves *et al.*, 1973; Bonney *et al.*, 1974; Wilson & Sharp, 1975b; Davies & Collins, 1979) and from chicken pituitary tissue *in vitro* (Tanaka *et al.*, 1974; Bonney & Cunningham, 1977a; Chou *et al.*, 1985), and also induces ovulation in laying hens (van Tienhoven & Schally, 1972; Reeves *et al.*, 1973).

Two molecular forms of GnRH have been extracted from the chicken brain and designated [Gln^8]-GnRH (GnRH-I; King & Millar, 1982a, 1982b; Miyamoto *et al.*, 1982, 1983; FIGURE 1.2) and [His^5, Trp^7, Tyr^8]-GnRH (GnRH-II; Miyamoto *et al.*, 1984; FIGURE 1.2).

mGnRH	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2
chicken GnRH-I	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-**Gln**^{8}-Pro^9-Gly^{10}-NH_2
chicken GnRH-II	pGlu^1-His^2-Trp^3-Ser^4-**His**^{5}-Gly^6-**Trp**^{7}-**Tyr**^{8}-Pro^9-Gly^{10}-NH_2

FIGURE 1.2: Molecular structures of mammalian and chicken gonadotrophin-releasing hormones.
Highlighted amino acids indicate substituted amino acid differences from mammalian GnRH (mGnRH).

1.3 The hypothalamic control of LH secretion

1.3.1 Structure and function of the hypothalamus

The hypothalamus can be divided into the rostral (preoptic and supraoptic) and caudal (containing the infundibular nuclear complex) regions. The availability of antisera to GnRH-I and GnRH-II has enabled the mapping of GnRH neurones in the brains of chickens, turkeys and quail (Mikami *et al.*, 1988; Millam *et al.*, 1989; Katz *et al.*, 1990; Sharp *et al.*, 1990). Cell bodies containing GnRH-I are found in the preoptic, septal and dorsomedial areas of the hypothalamus, and have terminals in the median eminence (ME) and the organum vasculosum of the lamina terminalis (Mikami *et al.*, 1988). The function of GnRH nerve endings in the lamina terminalis is unknown but may communicate stimulatory information to the reproductive system (Sharp, 1983). The GnRH-I nerve terminals in the ME release GnRH-I into a capillary network covering its surface. These capillaries drain into specialised portal blood vessels to supply the anterior pituitary gland which is situated immediately below the ME (Sharp & Follett, 1969a). Transection of this humoral link causes regression of the ovary and demonstrates its functional significance in the control of reproduction (Shirley & Nalbandov, 1956).

GnRH-II is distributed more widely in the avian brain than GnRH-I but is present in smaller quantities (Mikami *et al.*, 1988; Sharp *et al.*, 1990). Cell bodies containing GnRH-II occur in the dorsal-rostral area of the hypothalamus but do not appear to send projections to the ME since GnRH-II is absent from the ME (Mikami *et al.*, 1988; Sharp *et al.*, 1990). The differential distribution of GnRH-I and GnRH-II in the avian brain suggests different functions. Although GnRH-II is more potent than GnRH-I in terms of its ability to release LH (Sharp *et al.*, 1987; Wilson *et al.*, 1989), evidence to date suggests that only GnRH-I is of physiological importance. Thus only GnRH-I is present in the median eminence (Mikami *et al.*, 1988; Sharp *et al.*, 1990) and GnRH-I but not GnRH-II is released in response to depolarisation of the isolated median eminence (Katz *et al.*, 1990; in Sharp *et al.*, 1990). Active immunisation of hens against GnRH-I, but not GnRH-II, reduces the concentration of plasma LH and produces ovarian regression (Sharp *et al.*, 1990). However, there is evidence emerging for the interaction of GnRH-II terminals with GnRH-I neurones in the anterior hypothalamus (Wilson *et al.*, 1990a, 1991). It has been concluded from these morphological and functional studies, that GnRH-I is the physiologically significant LH-releasing peptide, and despite the greater potency for releasing LH, GnRH-II is not directly involved in the control of LH.

1.3.2 Steroidal feedback on GnRH-I and GnRH-II neurones

Gonadectomised chickens secrete more LH than intact birds (Wilson & Sharp, 1975c), and this increased secretion is associated with elevated concentrations of GnRH-I but not of GnRH-II in the posterior hypothalamus (Knight *et al.*, 1983; Lal *et al.*, 1990; Sharp *et al.*, 1990; Wilson *et al.*, 1990b). The effects of gonadectomy on plasma LH and

hypothalamic GnRH-I concentrations are reversed by treatment with testosterone (Knight *et al.*, 1983) or 17ß-oestradiol (Wilson *et al.*, 1990b). Castration increases the basal and K^+-depolarisation-releaseable GnRH-I from perifused MBH tissue *in vitro* (Lal *et al.*, 1990). GnRH-II is not affected by castration or by treatment with testosterone or 17ß-oestradiol (Sharp *et al.*, 1990; Wilson *et al.*, 1990b). These observations indicate that steroids act directly at the level of the hypothalamus to regulate the release of GnRH-I.

Plasma testosterone is believed to be the negative feedback signal for the regulation LH secretion in male birds. However, this action of testosterone is mediated at least in part by its conversion into active metabolites at the level of the hypothalamus. Testosterone can be metabolised to 17ß-oestradiol, 5α-dihydrotestosterone (5α-DHT) and 5ß-DHT respectively by aromatase, 5α-reductase and 5ß-reductase activity. Aromatase activity is present in the brain and anterior pituitary gland of grouse and the quail (Sharp *et al.*, 1986a; Callard *et al.*, 1990), and in the cockerel, the inhibitory action of testosterone is mediated by 17ß-oestradiol (Wilson *et al.*, 1983). Thus administration of the anti-oestrogen tamoxifen which can pass the blood-brain barrier, but not the administration of antibodies to oestrogen, result in increased LH secretion in adult cockerels. Of the 5α and 5ß-reduced products of testosterone in the hypothalamus of the chicken (Massa & Sharp, 1981, 1985), only the administration of 5α-DHT reduces the concentration of plasma LH in the castrated quail (Davies *et al.*, 1980). Since 5ß-DHT has no effect on plasma LH in quail and chickens, the high 5ß-reductase activity in these tissues may represent an inactivation pathway for testosterone (Davies *et al.*, 1980; Massa & Sharp, 1981, 1985).

Unlike testosterone or 17ß-oestradiol, progesterone exerts a positive feedback action on LH release in the laying hen (see SECTION 1.3.3).

There is substantial evidence that the avian hypothalamus contains steroid receptors. Thus early studies using radiolabelled steroids showed specific binding sites for androgens (Stern, 1972; Zigmond *et al.*, 1972), 17ß-oestradiol (Gasc *et al.*, 1980; Kawashima *et al.*, 1987) and progesterone (Kawashima *et al.*, 1978, 1979, 1980) in the hypothalamus. Receptors for these steroids have also been demonstrated in the hypothalamus by immunocytochemical studies (Stumpf *et al.*, 1983; Sterling *et al.*, 1984a, 1987; Gahr & Hutchison, 1992) and by Scatchard analysis of hypothalamic homogenates (Kawashima *et al.*, 1987, 1989, 1992c). However immunocytochemical studies show that progesterone receptors do not occur in GnRH neurones of the chicken (Sterling *et al.*, 1987). There is no direct evidence for the presence of androgen or oestrogen-receptors in avian GnRH neurones. Apart from one report of a low proportion of GnRH neurones containing oestrogen-receptors in the guinea-pig (0.2%; Watson *et al.*, 1992), most studies agree that steroid receptors are not found in GnRH neurones (Fink, 1986; Fox *et al.*, 1990; Watson *et al.*, 1992). It can be concluded that GnRH neurones are regulated by steroid-receptive neurones which abut the GnRH cells. The nature of the neurotransmitters in these neurones remains to be established. Many neurotransmitter systems have been examined

for their anatomical organisation and their GnRH-releasing properties however the monoaminergic and opiatergic pathways have been studied in most depth.

1.3.2.1 Monoaminergic and opiatergic mediators of GnRH release

Nerve fibres containing 5-hydroxytryptamine, dopamine or noradrenaline are present in the chicken hypothalamus (Sharp & Follett, 1968; Knight *et al.*, 1982a, 1982b, 1984; Sharp *et al.*, 1984, 1989; Contijoch *et al.*, 1992) and many are located in the region of the GnRH-I neurones (SECTION 1.3.1). Administration of metabolic precursors of these monoamines modify the concentration of plasma LH by altering the secretion of GnRH-I (Knight *et al.*, 1982b, 1984; Sharp *et al.*, 1989).

Pharmacological studies have shown that dopamine and 5-hydroxytrypamine act within the hypothalamus to suppress the preovulatory release of LH in the hen (Knight *et al.*, 1982b, 1984; Sharp *et al.*, 1984, 1989). In contrast, noradrenaline and adrenaline could be stimulatory neurotransmitters in the induction of GnRH-I release. Thus blockade of α-adrenoceptors reduces the magnitude of the preovulatory surge of LH (Knight *et al.*, 1982b). Furthermore noradrenaline and adrenaline stimulate the release of GnRH from the isolated median eminence of quail and chickens (Millam *et al.*, 1984; Contijoch *et al.*, 1990). Together these observations show that the GnRH-I nerve terminals of the median eminence may be influenced by incoming monoaminergic pathways to modulate GnRH release (Knight *et al.*, 1982b, 1984; Sharp *et al.*, 1984, 1989; Contijoch *et al.*, 1992).

Opiate-containing neurones form part of the circuitry which control or modulate the release of GnRH from the mammalian median eminence by activating an inhibitory dopaminergic mechanism (reviewed by Kalra & Kalra, 1984; Rasmussen, 1991). Whether this opiate pathway exists in the chicken is uncertain because of conflicting observations between studies *in vivo* (Harvey & Scanes, 1987; Lal *et al.*, 1990) and *in vitro* (Stansfield & Cunningham, 1987a, 1987b, 1988; Lal *et al.*, 1990). Central to this disagreement is that the opiate antagonist naloxone should block the supposed sustained inhibitory tone of endogenous opioid peptides on GnRH neurones to elevate the release of GnRH and LH. However the antagonist increases GnRH release from the isolated cockerel MBH (Stansfield & Cunningham, 1987a, 1987b, 1988) without a corresponding rise in plasma LH (Harvey & Scanes, 1987; Lal *et al.*, 1990).

Thus there are monoaminergic and opiatergic pathways, GnRH-I neurones, and receptors for gonadal steroids within the confines of the hypothalamus. As mentioned in SECTION 1.3.2, there are no progesterone receptors in GnRH-containing cells, however progesterone receptors are found in close proximity to the cell nuclei of these peptidergic neurones (Sterling *et al.*, 1984). The identity of these cells is not known but potential candidates could be the monoaminergic and opiatergic neurones.

1.3.3 Steroidal control of the preovulatory release of LH in mammals and birds

There are important differences between mammals and birds with respect to the actions of gonadal steroids in the regulation of the concentration of plasma LH. In mammals, the negative feedback effects of 17ß-oestradiol and progesterone on the hypothalamic-pituitary system maintains the low concentration of plasma LH during most of the ovulatory cycle (reviewed by Fink, 1988). However development of the preovulatory surge of LH is achieved through the positive feedback effects of 17ß-oestradiol and progesterone. The precise mechanism of how this feedback develops is not clear but involves the release of GnRH from the hypothalamus and principally, increases in the sensitivity and responsiveness of the pituitary gland to GnRH. Initiation of the surge of GnRH is induced by the elevation of the concentration of plasma 17ß-oestradiol. Meanwhile the pituitary gland becomes sensitised to the conditions of rising 17ß-oestradiol and the release of GnRH. Progesterone may synergise with 17ß-oestradiol to enhance the secretion of LH from the pituitary gland and the generation of an LH surge.

In the laying hen, 17ß-oestradiol does not stimulate LH release, and instead progesterone induces the preovulatory release of LH (reviewed by Sharp, 1980; Cunningham *et al.*, 1984). Early studies showed that injection of progesterone into the hypothalamus, but not into the anterior pituitary gland, induced ovulation in hens (Ralph & Fraps, 1960). It is now known that progesterone releases GnRH-I from the posterior hypothalamus to produce a preovulatory release of LH and ovulation (Cunningham *et al.*, 1984; Wilson *et al.*, 1990a). Ovariectomy prevents the progesterone-induced surge of LH, and 17ß-oestradiol is therefore required to prime the hypothalamic-pituitary unit to the positive feedback effect of progesterone (Wilson & Sharp, 1976b). There is some evidence that in addition to stimulating GnRH-I release, progesterone acts to facilitate LH release directly from the anterior pituitary gland of laying hens (Kawashima *et al.*, 1982, 1992c).

The hypothalamic progesterone receptors in the chicken (Kawashima *et al.*, 1980; Stumpf *et al.*, 1983; Sterling *et al.*, 1984a, 1987), do not co-localise with GnRH-containing neurones although the receptors are present in nearby cells (Sterling *et al.*, 1984a, 1987). Similar observations have been described in the rat (Fox *et al.*, 1990). In view of the ability of progesterone injection into the hypothalamus to induce the preovulatory LH surge and ovulation (Ralph & Fraps, 1960; Sharp, 1980), it has been suggested that progesterone may act on other neurones which directly or indirectly impinge on the GnRH-cells thereby affecting GnRH release (Sterling *et al.*, 1987; Fox *et al.*, 1990), or that progesterone combines with cell membrane-associated progesterone receptors (Schumacher, 1990; Meizel & Turner, 1991) which may be undetectable by the antiserum preparation (Tuohimaa *et al.*, 1984; Sterling *et al.*, 1984a, 1987).

1.3.4 The mode of GnRH release

LH in mammals is secreted in a pulsatile pattern (Dierschke *et al.*, 1970; reviewed by Knobil, 1992) corresponding temporally with the episodic release of GnRH into the hypophyseal portal supply (Wildt *et al.*, 1981; Clarke & Cummins, 1982; Levine *et al.*, 1982; Barrell *et al.*, 1992). Thus, GnRH neurones are activated synchronously for a matter of minutes to release GnRH and generate an episode of LH secretion (reviewed by Dyer & Robinson, 1989; Knobil, 1989). It is assumed that the episodic pattern of LH release measured in cockerels (Wilson & Sharp, 1975c), male turkeys (Bacon *et al.*, 1991) and Japanese male quail (Gledhill, 1977) derives from a similar pattern of GnRH secretion. In contrast in the laying hen, apart from the preovulatory release of LH, the LH does not appear to be secreted in a pulsatile manner. However LH secretion is pulsatile in ovariectomised hens suggesting that any pulsatile release of LH in the intact hen is suppressed by an inhibitory action of ovarian steroids to the extent that they are too small to be detected (Wilson & Sharp, 1975c).

The human mediobasal hypothalamus (MBH) continues to discharge GnRH in pulses when the tissue is maintained in a perifusion apparatus (Rasmussen *et al.*, 1989) suggesting that the MBH region possesses all the necessary neural circuitry to retain intrinsic GnRH pulsatility. Similar studies on the cockerel MBH show no evidence of an inherent pacemaker for GnRH secretory episodes (Knight, 1983; Stansfield & Cunningham, 1987b; Lal *et al.*, 1990). This means that the 'pacemaker' activity of the chicken lies outside the region of the MBH and therefore relies on incoming neuronal afferents projecting from elsewhere in the brain (SECTION 1.3.2.1).

1.4 The anterior pituitary gland-gonadal axis

1.4.1 Anatomy of the anterior pituitary gland

The anterior pituitary gland of the chicken comprises two portions, the cephalic and caudal lobes. Studies on the cytology and ultrastructure of the avian pituitary gland have identified a number of different secretory cell-types (Tai, 1976; Marchand & Sharp, 1977; Tai & Chadwick, 1977; Sharp *et al.*, 1979), including the gonadotroph cells. The principal function of gonadotroph cells is to manufacture and secrete LH and FSH. These cells are distributed throughout the pituitary gland (Marchand & Sharp, 1977; Noce *et al.*, 1989) although the proportion in which they are represented is not known. Electron microscopy studies reveal two classes of chicken gonadotroph cells designated Types III and IV, suggested to contain FSH and LH respectively (Tai, 1976; Tai & Chadwick, 1977). This is consistent with the one cell-one hormone concept as observed in bovine gonadotroph cells (Bastings *et al.*, 1991). However LH and FSH co-exist within the same cell in rat pituitary glands (Lloyd & Childs, 1988; Childs, 1991).

1.4.2 Structure and functions of FSH and LH

The gonadotrophins are glycoproteins which comprise two non-covalently bound subunits. The α-subunit is common to both hormones (reviewed by Pierce & Parsons, 1981; Wilson *et al.*, 1990d). The ß-subunit confers the unique biological properties to each hormone (reviewed by Pierce & Parsons, 1981; Wilson *et al.*, 1990d). Each protein subunit is synthesised by separate genes (reviewed by Pierce & Parsons, 1981), and the two subunits are linked together by oligosaccharides during the later stages of hormone processing, and the packaging of the gonadotrophins into secretory granules by the Golgi complex (Hubbard & Ivatt, 1981; Hurbani-Kosnath *et al.*, 1987).

Luteinizing hormone has two functions. First in both sexes it regulates steroidogenesis, the synthesis and secretion of progesterone, testosterone and 17ß-oestradiol. Secondly, in the adult female it induces ovulation. The first of these functions involves the maintenance of the 'tonic' secretion of LH by the inhibitory feedback effects of gonadal steroids. The preovulatory surge of LH is generated by the positive feedback action of 17ß-oestradiol or progesterone (SECTION 1.3.3) on GnRH and LH release.

The ovulation-inducing property of LH has been demonstrated in the chicken by injection of the hormone (Kamiyoshi & Tanaka, 1972; Imai, 1973) and by showing that administration of anti-LH serum blocks ovulation (Sharp *et al.*, 1978). Studies in the chicken *in vivo* (Shahabi *et al.*, 1975a) and *in vitro* (see below) show that LH is steroidogenic and that the steroid released depends on the maturity of the developing ovarian follicles. Thus LH stimulates progesterone secretion from granulosa cells (Marrone & Hertelendy, 1983; Robinson *et al.*, 1988) and 17ß-oestradiol and testosterone from theca cells (Marrone & Hertelendy, 1983; Robinson & Etches, 1986) of large preovulatory follicles. Smaller follicles (<10 mm) secrete oestrogens, dehydroepiandrosterone and androstenedione (Robinson & Etches, 1986; Robinson *et al.*, 1988). FSH stimulates a low release of progesterone from the less mature follicles, but not from the large preovulatory follicle (Hammond *et al.*, 1981; Robinson *et al.*, 1988) and is therefore probably not an important steroidogenic hormone in the hen (Robinson *et al.*, 1988). However, FSH augments the steroidogenic potency of LH in the chicken ovary (Kamiyoshi *et al.*, 1988). This suggests a supportive rather than an inductive action of FSH on steroidogenesis in the chicken.

An increase in plasma LH stimulates testosterone secretion in adult cockerels (Sterling *et al.*, 1978) and adult turkey toms (Guémené & Williams, 1992b). LH also stimulates the secretion of testosterone from testicular fragments of quail and cockerels *in vitro* (Maung & Follett, 1977; Chase, 1982) and increases the number of Leydig cells (Brown *et al.*, 1975). FSH does not stimulate testosterone secretion *in vivo* or from avian testes *in vitro* (Chase, 1982) but is required to promote testicular growth and spermatogenesis (Brown *et al.*, 1975; Follett, 1976; Ishii & Yamamoto, 1976; Brown & Follett, 1977; Desjardins &

Turek, 1977). In the hen, FSH promotes follicular growth and increases the number of granulosa cells (Yoshimura & Tamura, 1988).

1.5 Effects of gonadal steroids on LH release

1.5.1 Sex differences in the release of LH in response to GnRH

There are sex differences in the baseline concentration of plasma LH and the LH response to injection of GnRH in the chicken. These are described below with particular emphasis on the involvement of gonadal steroids in the regulation of LH secretion.

1.5.1.1 Sexually differentiated patterns of plasma LH

Comparisons of the resting levels of plasma LH between the adult sexes in chickens (Sterling & Sharp, 1984; Sharp *et al.*, 1987) and quail (Nicholls *et al.*, 1973; Davies, 1976) show lower concentrations in females than in males. The difference in the concentrations of plasma LH between the juvenile (9-week old) cockerel and hen are less pronounced than in the adult sexes (Wilson *et al.*, 1989). The concentration of LH in adult rats also appears to be sexually differentiated, with concentrations of 1 - 31 ng/ml in the male (Debeljuk *et al.*, 1974, 1975; Fink & Henderson, 1977) and values of between 1 and 50 ng/ml in the female outside the preovulatory release of LH (Aiyer *et al.*, 1974b; Fink & Henderson, 1977; Evans *et al.*, 1983).

1.5.1.2 Steroid-induced changes in sensitivity to GnRH

The relative potency of GnRH-II for releasing LH is 36.5-times greater than GnRH-I in laying hens, whereas the two LH-releasing hormones are equipotent in adult cockerels, but the laying hen is less sensitive to these hormones than is the male (Sharp *et al.*, 1987). Furthermore, a relationship can be established between the dose of GnRH-II (but not of GnRH-I) and the LH response in laying hens (Sharp *et al.*, 1987; Wilson *et al.*, 1989). In contrast, the adult cockerel (Sharp *et al.*, 1987) and the juvenile sexes express dose-related increases in LH secretion in response to both peptides (Wilson *et al.*, 1989).

The sensitivity of rat gonadotroph cells to GnRH may relate to the number of GnRH-receptors or the affinity for its ligand (Zilberstein *et al.*, 1983), or depend on the efficacy of post-receptor pathways involved in the GnRH-stimulated release of LH (see SECTION 1.6.1; Smith *et al.*, 1983; Gorospe & Conn, 1987a, 1987b, 1988; Chang *et al.*, 1988a). Steroids can modify the sensitivity of gonadotroph cells to GnRH. Thus, long-term treatment of mammalian pituitary cell cultures with 17ß-oestradiol (4-days) reduces the ED_{50} (effective dose which produces half-maximum stimulation) of GnRH on LH release (Drouin *et al.*, 1976; Tang *et al.*, 1982a) and is associated with an increase in the number of GnRH binding sites (Tang *et al.*, 1982a) and GnRH-receptor-mRNA (Sealfon *et al.*, 1990). The converse is seen after short-term (<4-hours) treatment with 17ß-oestradiol (Emons *et al.*, 1988). Furthermore, treatment with 5α-DHT reduces both the release of

LH and the number of binding sites for GnRH (Giguere *et al.*, 1981). GnRH can also up- and down-regulate its own pituitary receptors in the rat both *in vivo* and *in vitro* (reviewed by Catt *et al.*, 1985; Clayton *et al.*, 1985; Clayton, 1989). The physiological importance of the steroid-GnRH receptor relationship is indicated by the changes in GnRH-receptor number during the oestrous cycle (Clayton *et al.*, 1985), but these are suggested to represent an *indirect* contribution from the cyclic changes in GnRH secretion (Sarkar *et al.*, 1976). More recently however, it has become evident that the number of GnRH-receptors is not always related to the sensitivity of gonadotroph cells to GnRH (Abbot *et al.*, 1986; Clarke *et al.*, 1988).

The direct effect of steroids on chicken gonadotroph function is unlikely to be mediated by an action on GnRH-receptors. Functional evidence for this is that gonadal steroids do not affect the ED_{50} of GnRH-I on LH release (King *et al.*, 1989). Thus, 17ß-oestradiol treatment does not appear to change the GnRH-receptor number or affinity for GnRH-I of cultured chicken pituitary cells (King *et al.*, 1989). Other than this report for the absence of a functional change in GnRH-receptors, there is no *direct* evidence to support this claim due to the lack of a sensitive and reliable receptor assay for characterising GnRH binding sites in chicken pituitary tissue.

1.5.1.3 Sex differences in the magnitude of GnRH-stimulated LH secretion

Adult cockerels release more LH in response to a maximal dose of GnRH than laying hens. This is seen after injection of GnRH-I, GnRH-II (Sharp *et al.*, 1987), mGnRH or the GnRH superagonist buserelin (Sterling & Sharp, 1984). The responsiveness of 9-week old hens to GnRH-I or GnRH-II is less than that of the age-matched cockerel (Wilson *et al.*, 1989) but this sex difference is less marked than it is in adult chickens. The LH responsiveness of adult Japanese quail to mGnRH is also sexually differentiated. Thus the adult female releases less LH than the adult male quail, but the juvenile sexes show no difference in their responses to mGnRH (Davies, 1976).

There is a sex difference in the magnitude of the LH response to GnRH in the adult rat. GnRH increases the concentration of plasma LH from 3 to 18 ng/ml in adult males (Fink & Henderson, 1977), and from 25 - 50 to 156 - 600 ng/ml in the female rat, depending on the stage of the oestrous cycle (Blake, 1978; Pickering & Fink, 1979b). The pituitary concentration of LH in the diestrous female is only half the level in the male (Debeljuk *et al.*, 1975), but this is less during proestrous (Naftolin *et al.*, 1972). Continuous infusions of GnRH produce a far greater release of LH in women than in men, and these female responses are associated with the prevailing gonadal steroid environment of the menstrual cycle (Bremner & Paulsen, 1974; Hoff *et al.*, 1977). The LH responses to GnRH of pituitary tissue prepared from rats relative to their oestrous cycle also exhibit stage-specific changes in magnitude (Tang, 1978; Baldwin *et al.*, 1983; Evans *et al.*, 1983; Loughlin *et al.*, 1984), and correlate with the concentration of plasma 17ß-oestradiol during the

ovulatory cycle (Evans *et al.*, 1983). Pituitary glands from gonadectomised and steroid-supplemented rats also show pronounced changes in the secretion of LH (Baldwin *et al.*, 1983; Kitahara *et al.*, 1990). Finally, there are changes with respect to the oestrous cycle of the rat in the pituitary content of LH (Naftolin *et al.*, 1972; Blake, 1980), the amount of 'readily releaseable' LH in the pituitary gland (Pickering & Fink, 1979b), and the number of secretory granules per gonadotroph cell (Blake, 1980).

1.5.1.4 Sex difference in the profile of LH secretion

The profile of plasma LH after injection of GnRH is sexually differentiated in adult but not juvenile chickens. In adult cockerels and the juvenile sexes, GnRH-I or GnRH-II produce a sharp peak of plasma LH which then declines towards baseline levels of LH (Sharp *et al.*, 1987; Wilson *et al.*, 1989). The LH response of the laying hen differs in that neither GnRH-I or GnRH-II produce a distinct peak of plasma LH (Sharp *et al.*, 1987; Wilson *et al.*, 1989), and the duration of elevated LH is more prolonged than that of the adult cockerel (Sharp *et al.*, 1987).

The profile of LH release from adult rat pituitary tissue in response to GnRH is sexually differentiated. The female pituitary tissue secretes a sustained level of LH, but the male response is transient despite the continuous infusion of GnRH (Loughlin *et al.*, 1984). The sustained responses of the female are biphasic and composed of an initial phase lasting about 1-hour, and followed by a second period of secretion which persists for the duration of stimulation with GnRH (Baldwin *et al.*, 1983; Evans *et al.*, 1983). The relative sizes of each phase varies with the stage of the oestrous cycle (Baldwin *et al.*, 1983; Evans *et al.*, 1983). However, infusions of GnRH into men and women show similar biphasic profiles of plasma LH (Bremner & Paulsen, 1974; Hoff *et al.*, 1977).

1.5.2 Gonadal steroids and gonadotroph function

It is likely that steroids target the pituitary gland since it contains steroid receptors. Oestrogen-receptors are confined mainly to the gonadotroph cells of the chicken pituitary gland (Gasc *et al.*, 1980) localised in the cytosol and nucleus (Kawashima *et al.*, 1987). The presence of nuclear oestrogen-receptors indicates a genomic action of steroids to alter cell function. Receptors for progesterone and androgens are also found in the avian pituitary gland (Stern, 1972; Gasc *et al.*, 1979, 1980; Stumpf *et al.*, 1983; Sterling *et al.*, 1987; Kawashima *et al.*, 1992c) but their localisation within the gonadotroph cells has not been confirmed in birds as they have in mammals (Lloyd & Karavolas, 1975; Sar & Stumpf, 1979; Thieulant & Duval, 1985; Kamel & Krey, 1991).

The actions of steroids are mediated through interactions with their receptors located in the target cells. Classically, the gonadal steroids are released into the circulation and transported to their target tissues, where they are translocated into cells and combine with

specific steroid receptor proteins in the cytosol and then transported into the cell nucleus to bind to steroid regulatory elements which modulate gene expression and hence protein synthesis (Lebeau & Baulieu, 1975; Callard *et al.*, 1990). Evidence for the requirement to form new protein comes from the prevention of steroid action by actinomycin-D (Jackson *et al.*, 1973; Stryer, 1982). More recently, there is growing evidence for steroid receptors at the cell membrane, and their intracellular signalling pathways which mediate the immediate effects of steroids on cellular function (reviewed by Schumacher, 1990).

Oestrogen suppresses the concentration of plasma LH in chickens (Massa & Sharp, 1985; Wilson *et al.*, 1983, 1989, 1990b) and the GnRH-stimulated release of LH from chicken pituitary cells (Bonney & Cunningham, 1977d; Luck & Scanes, 1980; King *et al.*, 1989). However there is also evidence for an oestradiol-induced enhancement of GnRH-stimulated LH release *in vitro* from pituitary cells of turkeys (Knapp *et al.*, 1987) and chickens (Bonney & Cunningham, 1977d), and in laying hens *in vivo* (Bonney & Cunningham, 1977d). Testosterone also inhibits LH concentrations *in vivo* (Davies *et al.*, 1980; Wilson *et al.*, 1983; Massa & Sharp, 1985) and *in vitro* (Bonney & Cunningham, 1977d; Connolly & Callard, 1984; King *et al.*, 1989), but there is also evidence for a stimulatory effect of testosterone on LH secretion *in vivo* (Davies & Bicknell, 1976; Wilson & Sharp, 1976a; Wilson, 1978) from chicken (Luck & Scanes, 1980) and turkey pituitary cells (Knapp *et al.*, 1987). Progesterone similarly exhibits either positive (Wilson & Sharp, 1975b, 1976b; Kawashima *et al.*, 1982, 1992c; Knapp *et al.*, 1987) or negative actions on LH release *in vivo* and *in vitro* (Wilson & Sharp, 1975b, 1976b; Bonney & Cunningham, 1977d; King *et al.*, 1989). Consequently, a general consensus cannot be reached from the small number of investigations on the effect of gonadal steroids on avian gonadotroph function *in vitro*. They appear to be related to the use of different treatment periods, and the sex and age of the donor of pituitary glands.

1.5.3 Non-steroidal regulation of gonadotroph function

Hormones other than the gonadal steroids which affect gonadotroph function can be classified into the intrapituitary and extrapituitary factors. Inhibin is an FSH-specific inhibitor secreted by the gonads of mammalian species (reviewed by Ying, 1989; Robertson, 1992), however it has only recently become of research interest in birds. Large quantities of inhibin-immunoreactivity are present in the plasma and gonads of adult and late embryonic chickens (Rombauts *et al.*, 1992; Vanmontfort *et al.*, 1992; Johnson *et al.*, 1993), and there is a greater content of gonadal inhibin in adult cockerels than in laying hens (Rombauts *et al.*, 1992). Inhibin has been shown to be present in chicken granulosa cells (Akashiba *et al.*, 1988; Tsonis *et al.*, 1988; Johnson & Wang, 1992; Vanmontfort *et al.*, 1992), and this concentration of inhibin decreases with increasing maturity of the follicles (Vanmontfort *et al.*, 1992). Chicken inhibin specifically suppresses FSH secretion without affecting LH or prolactin release from sheep (Tsonis *et al.*, 1988) and rat

pituitary cells (Akashiba *et al.*, 1988). However, both gonadotrophins stimulate the secretion of inhibin from granulosa cells *in vitro* in response to FSH, and more potently, to LH (Vanmontfort *et al.*, 1992). The functional importance of inhibin in laying hens is indicated by the reduction in plasma inhibin induced by a loss of reproductive function (Vanmontfort *et al.*, 1992). Recently, the chicken inhibin α-subunit of the heterodimer has been cloned and sequenced, and shows approximately 60% homology with mammalian α-inhibin sequences (Johnson & Wang, 1992). Thus, inhibin is probably a significant modulator of gonadotroph function in the chicken, as it is in mammals.

Approximately thirty intrapituitary factors have been identified and are thought to 'fine-tune' the function of pituitary cells (reviewed by O'Halloran *et al.*, 1991; Schwartz & Cherny, 1992). Some of these factors (neuropeptide Y - Crowley *et al.*, 1990; substance P - Battmann *et al.*, 1991, Shamgochian & Leeman, 1992; PACAP [pituitary adenylate cyclase-activating peptide] - Canny *et al.*, 1992; oxytocin - Robinson *et al.*, 1992; melatonin - Vanecek & Klein, 1992) have potent or no effects themselves on rat gonadotroph cells, but can act in combination with GnRH to inhibit or stimulate gonadotroph activation. This *paracrine* control of gonadotroph function has not been investigated in avian species.

1.6 Mechanisms of GnRH-stimulated gonadotroph activation

Adult rat pituitary glands exhibit a sex difference in their LH responses to GnRH and gonadal steroids have been shown to modify these responses. These effects could indicate that the sex differences occur along some part of the stimulus-secretion coupling pathway used by GnRH. The basic components of this pathway are described to illustrate the sites at which the sex difference can occur, and how steroids affect the LH response to GnRH in this pathway.

1.6.1 Intracellular signalling mechanisms of GnRH-stimulated LH secretion

GnRH increases the activity of gonadotroph cells and the mechanisms involved in this process have been studied in mammals and the chicken. Interaction of GnRH (the *primary* messenger) with its receptors at the gonadotroph cell membrane triggers a series of *second messenger* pathways which amplify and communicate this activation signal into the cell to effect changes in function (reviewed by Conn *et al.*, 1987a; Davidson *et al.*, 1990; Naor *et al.*, 1990). These changes include the synthesis, processing and secretion of the gonadotrophins and the regulation of GnRH-receptors (reviewed by Clayton, 1989; Leong & Thorner, 1991). Only the effect on LH release has been studied in any depth in the chicken (reviewed by Davidson *et al.*, 1990). For a single peptide to exert a differential control over these actions, the gonadotroph cell is thought to interpret a 'code' of GnRH exposure to the cell, which includes the concentration of GnRH and the frequency of

stimulation (Leong & Thorner, 1991). Many messenger pathways are involved in LH secretion. One of these involves changes in the intracellular concentration of calcium ions.

A central role has been placed on Ca^{2+} in the activation of hormone secretion in many cell-types (reviewed by Putney, 1990), including the mammalian (reviewed by Stojilkovic & Catt, 1992) and chicken gonadotroph cell (Bonney & Cunningham, 1977b; Luck & Scanes, 1980; Davidson *et al.*, 1990; Johnson & Tilly, 1991). The actions of Ca^{2+} are mediated through calmodulin and calmodulin-dependent enzymes including adenylyl cyclase, cyclic nucleotide phosphodiesterases and protein kinases (Davidson *et al.*, 1987a; Hawes & Conn, 1990). Thus, GnRH elevates the intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$, in mammalian gonadotroph cells in association with LH release. This increased $[Ca^{2+}]_i$ comes from intracellular stores and from the extracellular medium (reviewed by Stojilkovic *et al.*, 1992b; FIGURE 1.3).

Intracellular Ca^{2+} is stored in an endoplasmic reticulum-like structure (Meldolesi *et al.*, 1988) or 'calciosome' (Volpe *et al.*, 1988), but has not been identified by electron microscopy. The presence of a releaseable store of intracellular Ca^{2+} has been demonstrated in pituitary cells of rats (Limor *et al.*, 1987; Naor *et al.*, 1988; Tanaka *et al.*, 1988; Stojilkovic *et al.*, 1989a) and juvenile chickens (Johnson & Tilly, 1991). Thus GnRH increases the $[Ca^{2+}]_i$ of rat gonadotroph cells maintained in a Ca^{2+}-free medium (reviewed by Stojilkovic & Catt, 1992) and releases $^{45}Ca^{2+}$ from rat (Hopkins & Walker, 1978) and chicken pituitary cells preloaded with $^{45}Ca^{2+}$ (Davidson *et al.*, 1987a, 1988; King *et al.*, 1989). The intracellular stores of Ca^{2+} are mobilised by inositol 1,4,5-trisphosphate (IP_3; Berridge, 1987; Naor *et al.*, 1988) which combine with its receptors on the membrane of the Ca^{2+} organelle (Guillemette *et al.*, 1987a). However this activity of IP_3 is short-lived and relies on its continuous production by the phospholipase C-mediated hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2; Schrey, 1985; Berridge, 1987; Morgan *et al.*, 1987; FIGURE 1.3).

Rat and chicken pituitary cells possess several classes of Ca^{2+} channel through which extracellular Ca^{2+} enters the cell. This is shown by the partial reduction in GnRH-stimulated and depolarisation-induced LH secretion using pharmacological blockers of voltage-sensitive Ca^{2+} channels (VSCC) such as nifedipine and nitrendipine (Smith *et al.*, 1987; Davidson *et al.*, 1988; reviewed by Stojilkovic & Catt, 1992). Agents which increase Ca^{2+} entry into gonadotroph cells by permeabilising cell membranes to Ca^{2+} also stimulate LH release (e.g. ionophore A23187, ionomycin; King *et al.*, 1986; Smith *et al.*, 1987; Davidson *et al.*, 1987a, 1988; Johnson & Tilly, 1991). The mechanism of Ca^{2+} channel activation is unclear because although gonadotroph cells possess voltage-sensitive Ca^{2+} channels, there are conflicting reports as to whether GnRH actually depolarises the cell membrane (Mason & Waring, 1985; Croxton *et al.*, 1988; Marchetti *et al.*, 1990). However, IP_3 can be phosphorylated to higher inositol polyphosphates (IP_4, IP_5, IP_6;

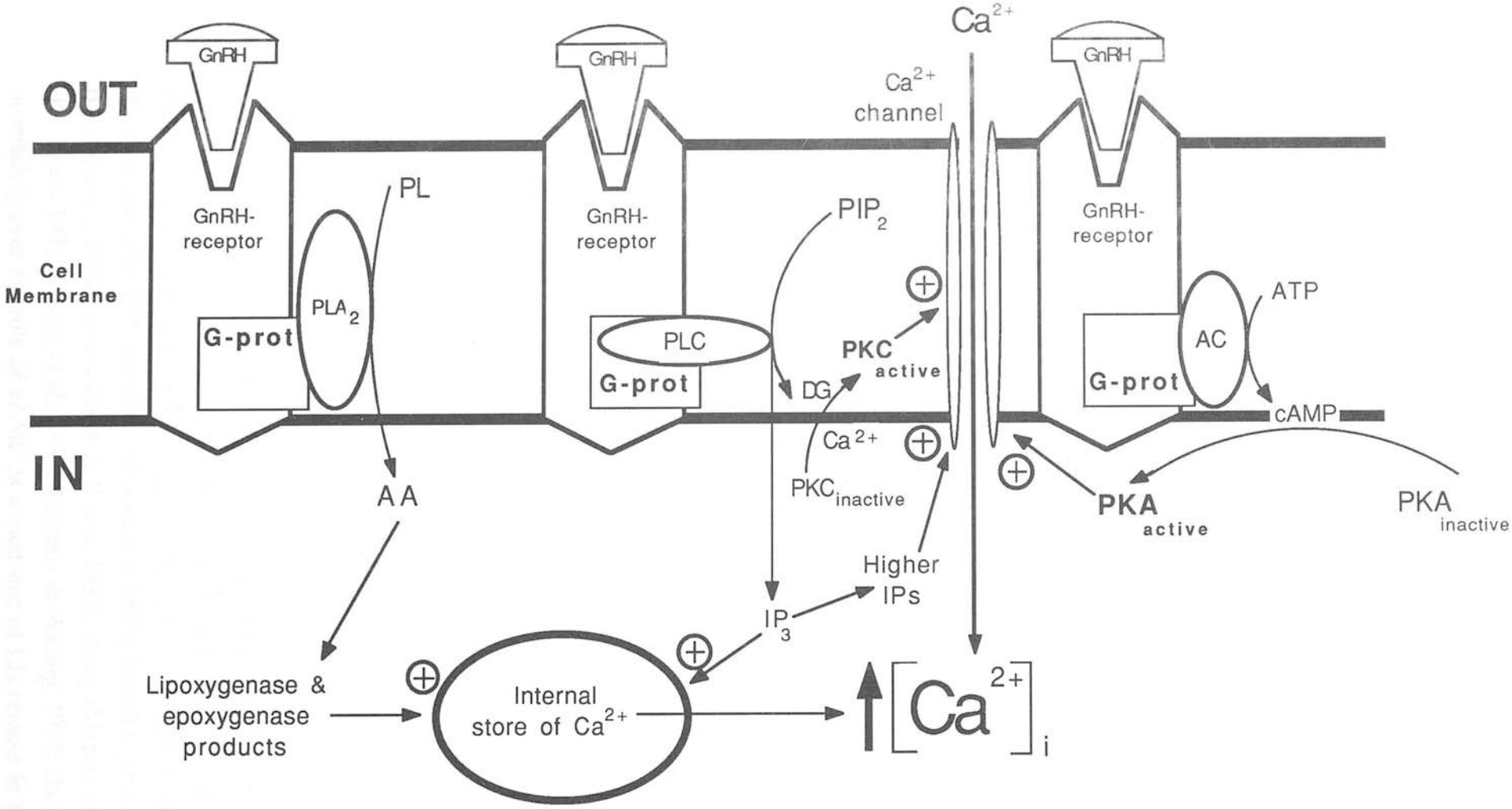


FIGURE 1.3: The principal intracellular signalling pathways of GnRH-stimulated LH secretion in the gonadotroph cell. ⊕ = stimulation

AA = arachidonic acid
AC = adenylyl cyclase
ATP = adenosine triphosphate
cAMP = cyclic 3'5'-adenosine monophosphate
DG = 1,2-diacylglycerol
G-prot = guanosine nucleotide-binding protein
IP3 = inositol 1,4,5-trisphosphate
PIP2 = phosphatidylinositol 4,5-bisphosphate
PKA = protein kinase A
PKC = protein kinase C
PL = phospholipid
PLA2 = phospholipase A2
PLC = phospholipase C

Guillemette *et al.*, 1987b; Morgan *et al.*, 1987) of which IP_4 and IP_6 act on specific receptors (Nicoletti *et al.*, 1990) to open non-voltage-sensitive Ca^{2+} channels in female rat pituitary cells (Sortino *et al.*, 1990), as well as other cells (reviewed by Berridge & Irvine, 1989).

The 'by-product' of PIP_2 hydrolysis is 1,2-diacylglycerol (DG) and together with Ca^{2+}, promotes the association of protein kinase C (PKC) with the cell membrane to activate the enzyme in secretory cells (reviewed by Nishizuka, 1984) including the rat and chicken gonadotroph cell (reviewed by Davidson *et al.*, 1990, Hawes & Conn, 1990; FIGURE 1.3). This effect of DG can be mimicked by tumour-promoting phorbol esters such as 12-O-tetradecanoyl 13-phorbol acetate (TPA) and phorbol dibutyrate (Nishizuka, 1984; Berridge, 1987) to stimulate the release of LH from rat (reviewed by Stojilkovic *et al.*, 1989a) and chicken pituitary cells (Davidson *et al.*, 1988; King *et al.*, 1989; Johnson & Tilly, 1991). In its activated form, PKC phosphorylates proteins which participate in secretion, the regulation of Ca^{2+} channels and GnRH-receptors, and in polypeptide synthesis (reviewed by Hawes & Conn, 1990, Stojilkovic *et al.*, 1992b).

Phospholipase C (PLC) is a cell membrane-bound enzyme and is probably coupled indirectly with the GnRH-receptor by a guanosine nucleotide-binding protein (G-protein; Andrews *et al.*, 1986; Ravindra & Aromstam, 1990, 1992b). These G-proteins act as transducers to communicate the signal of receptor activation to the cell interior (reviewed by Gilman, 1987; Birnbaumer *et al.*, 1991). Basically, receptor activation increases the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G-protein which then activates PLC (reviewed by Gilman, 1987; Birnbaumer *et al.*, 1991; FIGURE 1.3). Other enzymes which are linked to cell membrane receptors by G-proteins are adenylyl cyclase and phospholipase A_2.

Adenylyl cyclase catalyses the conversion of adenosine triphosphate (ATP) to cyclic 3'5'-adenosine monophosphate (cAMP; FIGURE 1.3) which activates a cAMP-dependent protein kinase (PKA). However, the role of adenylyl cyclase in GnRH-stimulated LH secretion in the pituitary gland of the rat has a controversial history. There are conflicting reports as to whether GnRH stimulates an increase in rat pituitary cAMP content (Adams & Nett, 1979; Conn *et al.*, 1979; Liu & Jackson, 1981b; Liu *et al.*, 1981; Cronin *et al.*, 1984; Bourne & Baldwin, 1987a, 1987b; Bourne, 1988) and there is also a considerable difference between the time-courses of cAMP accumulation and that of LH secretion (Borgeat *et al.*, 1972; Cronin *et al.*, 1984). However agents which elevate the concentration of cAMP potentiate the release of LH by GnRH in pituitary cells from fish (Chang *et al.*, 1992; Levavi-Sivan & Yaron, 1992), sheep (Macrae *et al.*, 1990) and rat (Liu *et al.*, 1981; Cronin *et al.*, 1984; Turgeon & Waring, 1986; Das *et al.*, 1991). The uncertainty over the role of cAMP as a mediator of LH release in the rat is therefore justified. In contrast, cAMP does seem to be important in the regulation of LH secretion

from pituitary cells of the chicken (Bonney & Cunningham, 1977a; Davidson *et al.*, 1990; Johnson & Tilly, 1991),

Phospholipase A_2 (PLA_2) represents a further route by which GnRH stimulates the gonadotroph cell (FIGURE 1.3). This enzyme liberates arachidonic acid from membrane phospholipids and this fatty acid, or one of its lipoxygenase or epoxygenase products (leukotrienes, hydroxyeicosatetraenoic acids), is considered to be a major mediator of GnRH-stimulated LH secretion in the rat (reviewed by Naor, 1990, 1991; Dan-Cohen *et al.*, 1992). Thus, exogenously added PLA_2 or arachidonic acid can release LH from chicken gonadotroph cells *in vitro* (Johnson & Tilly, 1991). The exact mechanism by which arachidonic acid releases LH is not known, but is suggested to promote the fusion of secretory granules with the cell membrane (Naor *et al.*, 1985a), or to mobilise intracellular Ca^{2+} directly (Chang *et al.*, 1987) or by enhancing the formation of IP_3 (Dan-Cohen *et al.*, 1992).

1.6.2 LH secretory mechanism

The processes of delivering secretory granules to the cell membrane and exocytosis itself are poorly understood. However the microfilaments and microtubules which make up the cytoskeleton undoubtably play a key role in the intracellular transport of secretory granules (reviewed by Ravindra & Grosvenor, 1990). The rise in intracellular Ca^{2+} induced by GnRH represents a vital step in the activation of this mechanism. In this respect, the Ca^{2+}-binding protein calmodulin and the calmodulin-dependent protein, caldesmon, have been shown to associate with secretory granules and the cytoskeletal apparatus in a Ca^{2+}-dependent manner in adrenochromaffin cells (Burgoyne, 1990) and rat pituitary cells (Janovick *et al.*, 1991).

1.6.3 Summary of intracellular signalling and LH secretion

The intracellular signalling pathways operate both in parallel and in a cascade formation which serve to *amplify* the effect of the GnRH stimulus and to *transform* these biochemical signals into a functional change in gonadotroph activity such as secretion. The activation of gonadotroph cells by GnRH is initiated by its interaction with GnRH-receptors. This signal is transduced through G-proteins to activate enzymes which stimulate PKA and PKC, and calmodulin-dependent protein kinases, and those which elevate the intracellular concentration of Ca^{2+} by mobilising internal stores and increasing Ca^{2+} entry through Ca^{2+} channels. The protein kinases function to phosphorylate proteins involved in the secretion and synthesis of gonadotrophins, and also the regulation of GnRH-receptors. From this account of the signal transduction pathways, it is clear firstly that there are many levels of the intracellular signalling cascade, and secondly that several of the critical events occur at the cell membrane. Consequently, interference at any part of

this pathway, particularly those at the cell membrane, will affect the cellular response to GnRH stimulation (Hirata *et al.*, 1980; King *et al.*, 1989; Kuomanov *et al.*, 1990).

1.6.4 Sex differences in the intracellular signalling mechanisms of LH secretion and their steroid-dependency

Studies have shown sex differences in the signalling mechanisms by which GnRH stimulates LH secretion, and also the association of these mechanisms with the action of gonadal steroids.

1.6.4.1 Sex differences in the Ca^{2+} requirements for LH secretion and their relationship with steroids

A sex difference in the extracellular Ca^{2+}-dependency of the GnRH-stimulated release of LH has been reported in the adult rat. GnRH increases LH secretion from female but not male pituitary tissue when maintained in a Ca^{2+}-free medium; this female response changes in magnitude in relation to the stage of the oestrous cycle (Baldwin *et al.*, 1983; Bourne *et al.*, 1988). The oestrogen-dependency of this extracellular Ca^{2+}-independent response is demonstrated by the fact that ovariectomy abolishes, and supplements of 17ß-oestradiol reinstate the GnRH-induced release of LH from pituitary glands of ovariectomised rats (Baldwin *et al.*, 1983; Bourne *et al.*, 1988). Likewise, pituitary glands from intact male or castrated rats implanted with 17ß-oestradiol respond to GnRH *in vitro* in the absence of Ca^{2+} (Bourne *et al.*, 1991). Since GnRH action requires an elevation of $[Ca^{2+}]_i$ for LH secretion (Stojilkovic *et al.*, 1992b), Ca^{2+} must originate from internal stores in the absence of an external source of Ca^{2+}. Whether there is a sex difference in the mobilisation of Ca^{2+} from this pool is not known. However, the rat pituitary gland shows no sex difference or steroid-induced change in the calmodulin-dependent proteins or calmodulin itself (Wooge & Conn, 1988), which means that the sex difference is present at a level of intracellular signalling subsequent to the activation of calmodulin.

The voltage-sensitive-Ca^{2+} channel agonist BAY K8644 stimulates the release of LH from pituitary cell cultures of adult female but not from those of adult male or ovariectomised female rats (Drouva *et al.*, 1988). However, treatment with 17ß-oestradiol for 72-hours restores or enhances the responses of pituitary cells prepared from respectively, ovariectomised or intact females (Drouva *et al.*, 1988). These observations show a sex difference in the basal activity of Ca^{2+} channels which is oestrogen-dependent. However, short-term (4-hours) treatments of cultures of female rat pituitary cells with 17ß-oestradiol *reduce* the LH responses to agents which depolarise cells to cause Ca^{2+} entry (e.g. veratridine, high K^+), and those which permeabilise cells to Ca^{2+} (e.g. ionomycin, ionophore A23187; Emons *et al.*, 1989). This indicates that 17ß-oestradiol does not act on Ca^{2+} channels, but at a step *after* the increase in $[Ca^{2+}]_i$ (Emons *et al.*, 1989). A study using pituitary cells from juvenile chicken supports this suggestion (King *et al.*, 1989). In addition, King *et al.* (1989) suggest that 17ß-oestradiol also acts at some level of signal

transduction *before* the mobilisation of intracellular Ca^{2+} because 17ß-oestradiol reduces the efflux of internal Ca^{2+} without affecting inositol polyphosphate production.

1.6.4.2 Role of steroids in the sex differences in cAMP-dependency of LH secretion

An oestrogen-dependent sex difference has been reported for the role of cAMP in LH secretion from adult rat pituitary tissue *in vitro*. Cultures of pituitary cells from adult females but not males, secrete LH and increase the content of LH in response to the cAMP analogue, dibutyryl cyclic AMP (dbcAMP; Tang, 1978), or the adenylyl cyclase-activator, forskolin (Kolp *et al.*, 1991). In addition, forskolin or GnRH increase the pituitary production of cAMP in both sexes, but only the female gonadotroph cells secrete LH in response to forskolin *in vitro* (Bourne & Baldwin, 1987a, 1987b; Kolp *et al.*, 1991). Furthermore, pituitary glands from ovariectomised rats lose their responsiveness to dbcAMP, and therefore express responses similar to that of the male (Tang *et al.*, 1982; Das *et al.*, 1991; Kolp *et al.*, 1991), however pretreatment with 17ß-oestradiol restores the response to dbcAMP (Tang *et al.*, 1982). In contrast with the female, GnRH does not increase the intracellular level of cAMP in male pituitary tissue maintained in Ca^{2+}-free medium (Bourne, 1988). This means that male but not the female rat gonadotroph cell requires extracellular Ca^{2+} in order to respond to a GnRH-stimulated activation of adenylyl cyclase.

Treatment of rat pituitary cells with gonadal steroids enhance or inhibit the secretion of LH in response to agents which elevate cAMP. Progesterone and 17ß-oestradiol show both effects depending on the period of exposure with the cells (Liu & Jackson, 1988; Krey & Kamel, 1990c; Kolp *et al.*, 1991), the positive effects of which are related to development of the preovulatory release of GnRH (SECTION 1.3.3). However testosterone suppresses the cAMP-stimulated release of LH (Drouin *et al.*, 1978). Furthermore, testosterone but not 17ß-oestradiol or progesterone, reduces the forskolin-induced release of LH in chicken pituitary cells (King *et al.*, 1989).

In contrast to the disputed role of cAMP in LH secretion (SECTION 1.10.1), its role is well-established in LH synthesis and the glycosylation of LH in the rat pituitary gland (Liu & Jackson, 1981b; Liu *et al.*, 1981; Counis *et al.*, 1988; Starzec *et al.*, 1989a, 1989b; Counis & Jutisz, 1991). Testosterone and 17ß-oestradiol (but not progesterone) modulate LH synthesis at the transcriptional and post-transcriptional levels. For example, these steroids regulate both the content of LHß-mRNA and common α-mRNA (Gharib *et al.*, 1986; Shupnik *et al.*, 1988, 1989; Simard *et al.*, 1988; Marshall *et al.*, 1990; Perheentupa & Huhtaniemi, 1990; Muyan & Baldwin, 1992; Weiss *et al.*, 1992; Winters *et al.*, 1992) and the synthesis and glycosylation of the polypeptide subunits (Ramey *et al.*, 1987a; Krummen & Baldwin, 1988). These steroidal effects are mediated by cAMP-dependent pathways utilised by GnRH (Counis *et al.*, 1988; Muyan & Baldwin, 1992).

1.6.4.3 Sex difference in pituitary G-proteins and their modulation by steroids

A detailed study has been made on the ability of 17ß-oestradiol to affect G-protein levels in the anterior pituitary gland of the rat (Bouvier *et al.*, 1991). Pituitary glands from adult female rats have a lower content of G-proteins than those from male rats (Bouvier *et al.*, 1991). In addition to the cyclic changes in pituitary G-protein levels corresponding to the oestrous cycle stage-specific concentration of plasma 17ß-oestradiol, administration of this steroid also depresses the elevated quantity of G-proteins in pituitary glands of ovariectomised rats (Bouvier *et al.*, 1991).

G-proteins possess intrinsic GTPase activity to inactivate its own transducing function (Gilman, 1987; Birnbaumer *et al.*, 1991). It has been shown that GnRH enhances, but co-incubation with 17ß-oestradiol, testosterone or progesterone suppress this GTPase activity (Ravindra & Aronstam, 1992a). Some pituitary G-proteins are functionally coupled to Ca^{2+} channels (Yatani *et al.*, 1987; Birnbaumer *et al.*, 1991) or enzymes (e.g. phospholipase C, adenylyl cyclase; Naor *et al.*, 1986; Cockcroft, 1987; Birnbaumer *et al.*, 1991; Mobbs *et al.*, 1991) to actuate an influx of Ca^{2+} or the generation of second messenger molecules. Gonadal steroids modify gonadotroph function by changing these G-protein - enzyme and G-protein - Ca^{2+} channel interactions, or by affecting enzyme activity directly (Bouvier *et al.*, 1991; Mobbs *et al.*, 1991; Ravindra & Aronstam, 1992a).

Steroids can evoke rapid cellular responses through steroid receptors at the cell membrane surface which are served by their own intracellular signalling pathways (Bression *et al.*, 1986; Morozova *et al.*, 1989; Emons *et al.*, 1989; Schumacher, 1990; Meizel & Turner, 1991; Morley *et al.*, 1992; Ravindra & Aronstam, 1992a). Evidence exists for membrane-associated steroid receptors in the rat pituitary gland (Bression *et al.*, 1986) and these receptors may be coupled to a G-protein transducer (Ravindra & Aronstam, 1992a). This indicates that in addition to affecting slow-onset genomic actions (40 - 60-minutes; Katzenellenbogen *et al.*, 1979), 17ß-oestradiol may also target the rapid coupling mechanism (seconds - minutes; Schumacher, 1990; Meizel & Turner, 1991; Morley *et al.*, 1992) by which GnRH activates the secretion of LH (Emons *et al.*, 1989; Ravindra & Aronstam, 1992a).

1.6.4.4 Steroids and protein kinase C

The secretion of LH from rat pituitary tissue stimulated with TPA is sexually differentiated and oestrogen-dependent (Bourne *et al.*, 1989; Fahmy *et al.*, 1989; Das *et al.*, 1991; Thomson *et al.*, 1993). Thus, pituitary glands from adult female rats release LH in response to TPA, but pituitary tissue from males and ovariectomised rats do not. Furthermore, supplements of 17ß-oestradiol allow LH to be released from pituitary tissue from these non-responding animals in response to TPA (Bourne *et al.*, 1989; Fahmy *et al.*, 1989; Das *et al.*, 1991).

The GnRH-I-induced formation of inositol polyphosphates in pituitary cells of juvenile chickens is unaffected by oestrogen-treatment (King *et al.*, 1989), and since PIP_2 is hydrolysed to form one molecule of IP_3 and one molecule of DG (Berridge, 1987), it can be deduced that DG production is probably also unaffected by 17ß-oestradiol. However, by activating PKC with TPA, only progesterone inhibits the release of LH; 17ß-oestradiol and testosterone having no effect on the LH response (King *et al.*, 1989). This compares with the bimodal effects of 17ß-oestradiol to either enhance the TPA-stimulated LH release in rat pituitary cells associated with an enhanced LH response to GnRH (Liu & Jackson, 1988, 1990; Fahmy *et al.*, 1989; Audy *et al.*, 1990; Thomson *et al.*, 1993), or the inhibition of TPA-stimulated LH release by 17ß-oestradiol associated with a reduced LH response to GnRH (Emons *et al.*, 1989). The potentiation of LH release by 17ß-oestradiol is related to the increased PKC activity in treated rat pituitary cultures, and in oestrogen-supplemented ovariectomised rats (Drouva *et al.*, 1990).

In addition to a role for PKC in LH secretion, a PKC-dependent mechanism is important in the GnRH-induced expression of the gonadotrophin genes in the rat (Andrews *et al.*, 1988; Counis *et al.*, 1988; Liu & Jackson, 1990; Leigh *et al.*, 1991). In this respect, 17ß-oestradiol enhances the rate of LH glycosylation *in vitro* (Liu & Jackson, 1990), progesterone treatment enhances the release of LH by TPA (Krey & Kamel, 1990c), however the dose-response curve to TPA in rat pituitary cells is unaffected by testosterone (Kamel & Kubajak, 1988).

1.6.4.5 Steroidal modulation of gonadotroph function by targetting PLA_2

Long-term (24 - 52-hours) treatment of female rat pituitary cell cultures with testosterone (Kamel & Kubajak, 1988) or 17ß-oestradiol (Liu & Jackson, 1988; Ortmann *et al.*, 1992a) do not affect the LH response to PLA_2, or the PLA_2-activator mellitin, or arachidonic acid. However, short-term (4-hours) incubations with 17ß-oestradiol depress both the GnRH-induced and the mellitin-stimulated release of LH (Ortmann *et al.*, 1992a). These reports indicate that steroids act on the signal transduction pathway used by GnRH, at a step preceding the release of arachidonic acid.

1.7 Summary and research objectives

GnRH-I stimulates sexually differentiated LH responses in the adult chicken however the differences are far less pronounced or absent between the juvenile sexes. The features of these responses are that compared with the adult cockerel, laying hens are both less sensitive and less responsive to the two peptides, but the resulting LH responses are more prolonged. The theme of this introductory chapter has been the association of gonadal steroids in sex differences in LH secretion. In this regard the high circulating concentration of 17ß-oestradiol in the laying hen could partly explain the sexually differentiated

responses of adult chickens. Evidence has also been reviewed here to indicate the possible actions of steroids on the intracellular signalling pathway used by GnRH.

The research objectives of this thesis were four-fold. The first was to further characterise the LH responses of adult and juvenile chickens to GnRH-I, and to determine the sex differences in the hypothalamic-pituitary-gonadal axis. The second objective was to determine the LH responses of the pituitary gland to GnRH-I *in vitro* by isolating it from the effects of circulating hormones and LH clearance. Thirdly, to establish the effects of 17ß-oestradiol on the pituitary gland and the response to GnRH-I. The final objective was to determine whether the sexually differentiated LH responses to GnRH-I *in vivo* were due to differences in the mechanisms of GnRH-I-stimulated secretion of LH.

2.1 GENERAL METHODS

2.1.1 Animals

Male and female White Leghorn (IAPGR, Roslin), ISA Brown (ISA Poultry Services, Forfar) or HISEX (Ross Poultry, Inverurie) chickens were reared from one-day-old chicks on a diet of LS mash or pellets for 4-weeks, when they were caged in groups of 16 (mixed sexes) and fed on LE mash or pellets. From 16-weeks of age, the birds were caged individually and fed LP mash or pellets. All day-old chicks were vaccinated against Marek's disease and received a booster after 3-weeks. At 4-weeks, all birds were vaccinated for Newcastle and Gumboro diseases and boosted at 16-weeks of age. Water was freely available through drinking nipples. Unless otherwise indicated, the daily lighting schedule was 14-hours light and 10-hours dark (lights on at 04:00-h GMT).

On the day of experiments using adult hens, only birds which had a good laying record and had laid an egg at least 3-hours previously were used (oviposition between 9:00 - 12:00-h; 5 - 8-hours after lights on). This was to avoid the preovulatory LH surge and the subsequent refractoriness of the pituitary gland to GnRH stimulation (Etches & Cunningham, 1976; Sharp, 1980). Juvenile chickens used in these studies were 6 to 8-weeks of age while adult birds were between the ages of 5 and 18-months. Twenty-one-week-old adult cockerels had fully developed testes (weights ranged between 20.2 - 35.3 g/2 testes) and the ductus deferens were distended with semen. Regular egg-laying sequences were recorded in the age-matched hens.

All suppliers of reagents and equipment are listed in APPENDIX 1.

2.1.2 Dosing and bleeds

All intravenous injections were administered into the brachial wing vein. A stock solution of GnRH-I (SIGMA or Peninsula Labs) was prepared in water and injected (100 µl/kg body weight)

in a vehicle of 0.9% (w/v) sodium chloride. Intramuscular injections (100 µl/kg body weight) of oestradiol benzoate (SIGMA) were given in arachis oil (SIGMA) into the pectoral muscle. Blood samples (1 ml) were withdrawn into heparinised (Evans Medical Ltd) syringes from the brachial wing vein and the plasma fractions obtained by centrifugation at 250 x **g**, 4°C for 15-minutes and stored at -20°C for later LH radioimmunoassay (RIA; SECTION 2.1.4.1).

2.1.3 Surgical and dissection procedures

2.1.3.1 Removal of hypothalamic and pituitary tissues

All chickens were killed by cervical dislocation. The sex of the juvenile birds was determined by comb size and confirmed subsequently by gross examination of the gonads.

There was a sex difference in the weights of pituitary tissue collected from chicken heads of sexually immature broilers. Male pituitary glands (10.9 ± 0.4 mg) weighed more than those of the female (7.8 ± 0.3 mg; $P<0.001$, n = 18/22). This was not due to the method for sexing the chicken heads (namely, comb height >10 mm = male, <10 mm = female) because FIGURE 2.1 shows a relationship established between comb height and sex in the juvenile broiler chickens.

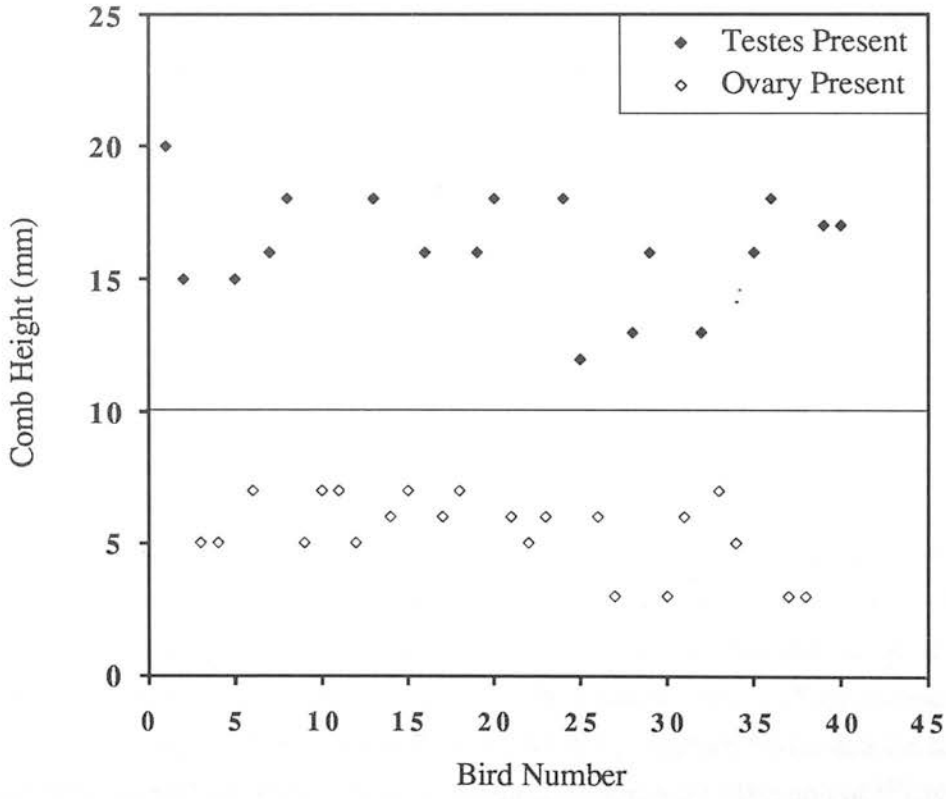


FIGURE 2.1: Validation of the 'comb height' method for sexing the juvenile chickens.
The comb heights of forty 7-week old juvenile broilers were measured and then their sex determined by inspection of the gonads. The horizontal line at 10 mm comb height indicates the demarcation size of comb used to sex the chickens.

Pituitary glands were also obtained from the heads of sexually immature (5 - 7-weeks of age) broiler chickens from a local poultry processing plant (Bibby's Chicken, Much Hoole, Preston) and transported back to the laboratory. The time between death and the removal of pituitary glands was typically less than one hour. Pituitary tissue obtained from this source were used for cell culture experiments (CHAPTER 6) and membrane fluidity studies (CHAPTER 7).

2.1.3.1.1 Hypothalamus

The ventral surface of the brain was exposed by gaining access through the roof of the oropharyngeal cavity. The optic chiasma was removed and cuts were made with a scalpel blade rostral to the preoptic area (POA), and to the roots of the oculomotor nerves. An area of tissue 2 mm either side of the midline was dissected free and divided into the mediobasal hypothalamus (MBH; includes the median eminence, ventromedialis hypothalami, and nucleus mamillaris medialis) and preoptic area (POA; includes the anterior commissure and the area immediately above and in front of it).

2.1.3.1.2 Anterior pituitary gland

The cranium was opened by inserting a pair of scissors into an opening of the skull at the exit of the spinal cord (*foramen magnum*), and making dorsal cuts through the parietal and frontal bones towards each eye. The 'lid' thus formed, was lifted up to expose the dorsal surface of the brain. The brain tissue was removed and the base of the cranium (*basioccipital condyle*, *basisphenoid* and *parasphenoid*) was cut from the *basioccipital condyle* towards the pituitary fossa. The skull was 'opened' along the line of this cut to reveal the anterior pituitary gland.

2.1.4 Hormone measurements

2.1.4.1 Radioimmunoassay of chicken LH

The double antibody method for radioimmunoassay of chicken LH was used in these studies (Sharp *et al.*, 1987).

2.1.4.1.1 Preparation of radiolabelled chicken LH

The chloramine T procedure for the iodination of purified chicken LH preparation, designated PRC-AE1-s-1 (Sharp *et al.*, 1987), was modified from Greenwood *et al.* (1963). All reagents were dissolved in 50 mM sodium phosphate buffer, pH 7.5, unless otherwise specified. A small stirring bar (short piece of paper clip) was added to the reaction vessel (1.5 ml microcentrifuge tube) containing 1.25 µg PRC-AE1-s-1 in 25 µl of 50 mM phosphate buffer and the iodination procedure performed with constant mixing by magnetic stirrer. A 37 MBq unit of ^{125}I[Na] (IMS-30, Amersham International plc) in 10 µl was introduced into the reaction tube and the iodination reaction started with 10 µl of freshly prepared 3.55 mM chloramine T sodium salt (Fisons). The reaction proceeded at room temperature (17 - 19°C) for 45-seconds and was terminated with 100 µl of 5.26 mM sodium metabisulphite (Fisons) and 100 µl of 0.6 M potassium iodide (Fisons). The

reaction mixture was transferred to a PD-10 Sephadex G-25 column (LKB-Pharmacia) which had been pre-equilibrated with column buffer; 50 mM phosphate buffer (pH 7.5) containing 0.2% (w/v) gelatine (Fisons). The reaction tube was rinsed with 200 µl column buffer and also added to the column. Column buffer was continuously supplied until 25 fractions of 10 drops had eluted into LP4 plastic tubes (Denley-Luckham Ltd). Radiolabelled PRC-AE1-s-1 was typically located between fractions 4 - 7 while the free iodine eluted between fractions 13 - 18. The label was diluted in RIA diluent to approximately 500,000 counts per minute (cpm) per 10 µl and stored for up to 4-weeks at 4°C.

Radioimmunoassay diluent (for use in assay of LH, GnRH-I and GnRH-II)

160 ml 0.5 M phosphate buffer, pH 7.5
17.5 g NaCl
5.84 g EDTA (disodium salt)
2 g sodium azide (FSA)
40 ml horse serum (GIBCO)

The volume was adjusted to 2 litres, pH 7.0.

2.1.4.1.2 Preparation of standards

Stock solutions of PRC-AE1-s-1 for the preparation of standards was stored at -20°C in 200 µl volumes of 250 ng/ml. A top standard of 5 ng/ml was prepared from one volume of stock PRC-AE1-s-1 diluted in 10 ml RIA diluent. Serial dilutions of 5 ml in 9 ml produced a final series of standards, designated Std_{10} - Std_1 at 5.0, 2.78, 1.54, 0.86, 0.48, 0.26, 0.15, 0.08, 0.05 and 0.03 ng/ml.

2.1.4.1.3 Radioimmunoassay procedure

The assay was set up in 6 x 50 mm RT20 (MacKay and Lynn) or LP2/2 (Denley-Luckham Ltd) plastic tubes using Hamilton MicroLab-M auto-dispensers (Howe and Co Ltd) as outlined in TABLE 2.1. Total, non-specific binding (NSB) and standard tubes were dispensed in triplicate and zero standards into 6 replicate tubes. Cockerel pooled plasma was routinely included in the assay for control purposes. Once the assay was set up, the tubes were vortex-mixed after each addition of reagent.

Stock solutions of rabbit anti-PRC-AE1-s-1 (designated anti-LH 3/3; Sharp *et al.*, 1987) were stored at -20°C at 1-in-190 dilution in 300 µl RIA diluent. A working concentration of 1-in-19,000 was prepared in diluent and added to the assay tubes (except Total and NSB tubes). After an overnight incubation at 4°C, approximately 12,000 cpm per 50 µl of ^{125}I-PRC-AE1-s-1 in diluent was added to all assay tubes and the assay returned to 4°C. Normal rabbit serum (NRS; Scottish Antibody Production Unit) and donkey anti-rabbit serum (DARS; Scottish Antibody Production

Unit) at 1-in-200 and 1-in-20 diluent respectively were each added to the assay tubes in 50 µl volumes on Day 3 (except Total tubes) and incubated at 4°C for at least 16-hours.

With the exception of the Totals, all assay tubes were centrifuged (MSE Coolspin or Sorvall RC-3B) at 1500 x **g** for 30-minutes at 4°C on Day 4. The integrity of the pellet was preserved by adding 50 µl of a 6% (w/v) suspension of soluble starch (Fisons) to each tube and re-centrifuging for a further 15-minutes. The supernatant fractions were aspirated to waste, and the Totals and pellets counted for 60-seconds on a gamma-counter (1277 GammaMaster; LKB-Pharmacia) and the data aquired into AssayZap™ (AssayZap Universal Assay Calculator; BIOSOFT).

The intra- and interassay variability were 6.4% and 9.8% respectively.

TABLE 2.1: Procedure for the radioimmunoassay of chicken LH.

TUBE	DAY1			DAY 2	DAY 3		DAY 4
	Std/Sample (µl)	Diluent (µl)	Anti-3/3 (µl)	Label (µl)	NRS (µl)	DARS (µl)	
Totals	NIL	NIL					
NSB	NIL	250					
Zero Std	NIL	200					
Std_1	200	NIL	50 µl per tube *		50 µl per tube **	50 µl per tube **	Centrifuge all tubes (except Totals) 1500 x g, 4°C, 30-min. Aspirate supernatents and count pellets for 60 s on gamma-counter
Std_2	"	"		50 µl per tube			
Std_3	"	"					
Std_4	"	"					
Std_5	"	"					
Std_6	"	"					
Std_7	"	"					
Std_8	"	"					
Std_9	"	"					
Std_{10}	"	"					
Control 1	5	195					
Control 2	10	190					
Control 3	20	180					
Unknown 1 Unknown 2 etc	Appropriate dilutions of samples in 200 µl						

* except Total and NSB tubes
** except Totals

2.1.4.2 Extraction and radioimmunoassay of GnRH-I and GnRH-II

The intra- and interassay variability were 6.4% and 9.8% respectively.

The procedure for the extraction and radioimmunoassay of chicken GnRH-I and GnRH-II was from Sharp *et al.* (1990).

2.1.4.2.1 Extraction of GnRH-I and GnRH-II

The brain areas were weighed on a torsion balance and immediately boiled for 10-minutes in 1 ml 2.0 M acetic acid. After cooling, the tissue was homogenised with a Teflon pestle and sonicated for 2-seconds ('Sonicator' Ultrasonic Liquid Processor; Heat Systems-Ultrasonics Inc). The solid material was removed by centrifugation at 13,000 x **g** (Biofuge A, Heraeus Sepatech) for 10-minutes and the supernatents centrifuged under vacuum at 40°C (Het-o-Vac, Inter-Med; Refrigerated Condensation Trap, Savant Instruments Inc.) and the residues reconstituted in 1 ml of RIA diluent before radioimmunoassay of GnRH-I and GnRH-II.

2.1.4.2.2 Iodination of GnRH-I and GnRH-II

GnRH-I and GnRH-II (both from Peninsula Laboratories) were labelled with ^{125}I using a modified chloramine T method of Greenwood *et al.* (1963).

Both peptides (0.9 µg/10 µl PBS) were reacted with ^{125}I in an identical fashion as chicken LH (SECTION 2.1.4.1.1), however the reactions were terminated after 2-minutes with 100µl of 0.19 M sodium metabisulphate and 100 µl of 0.1 M acetic acid. The iodinated peptides were separated on a reverse phase ODS HPLC column (250 mm x 4.6 mm) using a mobile phase of methanol (Rathburn Chemicals Ltd) and 20 mM ammonium acetate (Merck) in proportions of 50% (v/v) : 50% (v/v) for ^{125}I-GnRH-I, and 55% (v/v) : 45% (v/v) for ^{125}I-GnRH-II respectively (both adjusted to pH 4.0 with glacial acetic acid).

2.1.4.2.3 Radioimmunoassay of GnRH-I and GnRH-II

GnRH-I standards were prepared by seven 1-in-3 serial dilutions of a 2 ng/ml stock in RIA diluent to give 0.36, 1.09, 3.28, 9.88, 29.62, 88.88, 266.67 and 400 pg/tube (TABLE 2.2). Standards, quality controls (extract of pooled chicken brains) and unknowns were dispensed into RT20 or LP2/2 tubes in a volume of 200 µl and incubated overnight at 4°C with 50 µl 1-in-10,000 of rabbit anti-chicken GnRH-I (designated anti-GnRH-I 3/3; Sharp *et al.*, 1990). On Day 2, 50 µl of ^{125}I-GnRH-I containing 10,000 cpm was added to all the assay tubes. After an overnight incubation at 4°C, 50 µl normal rabbit serum (1-in-1200) and 50 µl donkey anti-rabbit precipitating serum (1-in-20) were added to the tubes and equilibrated for a further overnight period at 4°C. On Day 4, the assay was centrifuged at 1500 x **g** for 30-minutes at 4°C. The pellets were preserved by adding 50 µl of a 6% (w/v) suspension of soluble starch to each tube and re-centrifuging for a further 15-minutes. The supernatant fractions were aspirated to waste and the Totals and pellets were counted for 60-seconds on a gamma-counter and the data aquired into AssayZap™.

The procedure for the radioimmunoassay of GnRH-II was identical to the assay described for GnRH-I except that standards of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 pg/tube were used, and 1-in-240,000 diluted rabbit anti-chicken GnRH-II (designated anti-GnRH-II 10/2; Sharp *et al.*, 1990) and ^{125}I-GnRH-II replaced ^{125}I-GnRH-I. Intra and interassay variability for the GnRH-I RIA were 8.3% and 15.2%, and for the GnRH-II RIA, 4.2% and 8.0% respectively.

TABLE 2.2: Radioimmunoassay procedure for chicken GnRH-I and GnRH-II

TUBE	DAY 1			DAY 2	DAY 3		DAY 4
	Std/Sample (µl)	**Diluent (µl)**	**Antibody (µl)**	**Label (µl)**	**NRS (µl)**	**DARS (µl)**	
Totals	NIL	NIL					
NSB	NIL	250					
ZeroBlank	NIL	200					
Std_1	200	NIL	50 µl per tube *	50 µl per tube	50 µl per tube **	50 µl per tube **	Centrifuge all tubes (except Totals) 1500 x g, 4 °C, 30 min. *** Aspirate supernatents and count pellets for 60 s on gamma-counter
Std_2	"	"					
Std_3	"	"					
Std_4	"	"					
Std_5	"	"					
Std_6	"	"					
Std_7	"	"					
Std_8	"	"					
Std_9	"	"					
Control 1	Appropriate dilutions in 200 µl						
Control 2							
Unknown 1	"						
Unknown 2	"						
etc							

* except Total and NSB tubes which received diluent
** except Totals
*** 50 µl of 6% (w/v) soluble starch suspension in water is added and the tubes centrifuged for a further 15-min. The supernatent fraction is discarded and the pellets are counted for 60-s.

2.1.5 Extraction and radioimmunoassay of 17ß-oestradiol

Plasma 17ß-oestradiol was measured by the method of Webb *et al.* (1985) and consisted of an extraction procedure using a 17ß-oestradiol antibody-linked Sepharose, followed by a double antibody radioimmunoassay technique.

All test tubes and glassware, except the sintered glass columns, were washed in Milli-Q water (Millipore UK). Affinity chromatography columns were rinsed with 3 ml 90% (v/v) Distol-grade methanol (Fisons) in water, followed by 3 washes of 10 ml Milli-Q water. Residual liquid was removed with positive pressure applied to the columns and dried with tissue paper. Milli-Q grade water was used throughout the extraction and radioimmunoassay procedures.

2.1.5.1 Affinity chromatography extraction procedure

Ten microlitres of [2, 4, 6, 7, 16, 17-^{3}H]-oestradiol-17ß (Amersham International plc), equivalent to 1000 - 3000 dpm, was added to 16 x 125 mm screw-cap glass tubes containing 1 - 3 ml of sample or control plasma. The final volume was adjusted to 10 ml with water and each tube received 400 µl of 17ß-oestradiol antibody-linked Sepharose (Webb *et al.*, 1985). The capped tubes were continuously inverted (Denley-Luckham Ltd) overnight at room temperature. The

contents of each tube were decanted into sintered glass columns (10 mm diameter columns, glass sintered discs [porosity 1]; Schott Glass UK) and the tubes rinsed with 2 x 7 ml water. The sedimented antibody-Sepharose was rinsed with a further 7 ml of water and the excess water was removed with tissue paper and by applying slight positive pressure to the columns. The hormone was liberated by 3 ml 90% (v/v) methanol and collected into 16 x 125 mm glass tubes. Any residual liquid was retrieved by applying positive pressure to the columns. The eluate was evaporated to dryness under vacuum in a Buchler Vortex evaporator (Gallenkamp) at 40°C. Residues were reconstituted with 1.8 ml phosphate-buffered saline - gelatine (PBS-Gel; pH 7.5) and vortex-mixed at 40°C for 20-minutes before radioimmunoassay.

Phosphate-buffered saline - gelatine (PBS-Gel)

2000 ml 0.05 M phosphate buffer, pH 7.5
18 g NaCl
warm solution on heated magnetic stirrer
2 g gelatine
0.2 g thimerosal (SIGMA)

The efficiency of each extraction was determined by 5-minute count of 500 µl reconstituted hormone extracts in 4 ml OptiPhase-X scintillant (Fisons) by liquid scintillation counting (WALLAC 1410, LKB-Pharmacia). The counts were corrected for background and expressed as a percentage of the total counts for 10 µl of non-extracted [^{3}H]-oestradiol-17ß in 500 µl PBS-Gel and 4 ml scintillant.

2.1.5.2 Preparation of standards

Stock solutions of 17ß-oestradiol standard (SIGMA) were stored at -20°C as 500 pg/ml in PBS-Gel. A standard curve was set up for each radioimmunoassay as detailed in TABLE 2.3. Standards were prepared in triplicate 12 x 75 mm glass culture tubes (LIP) in a final volume of 500 µl.

2.1.5.3 Radioimmunoassay procedure

Hormone extracts were vortex-mixed thoroughly immediately before assay. Controls (pooled bovine plasma) and unknowns were dispensed in a final volume of 500 µl into duplicate tubes. Where dilution of the sample was necessary, this was prepared in PBS-Gel. Aliquots of the rabbit primary antibody (1-in-1000, designated R48, Webb *et al.*, 1985) were stored at -20°C and a working concentration of 1-in-40,000 was prepared in PBS-Gel. One hundred microlitres of ^{125}I-oestradiol-17ß (iodination described by Webb *et al.*, 1985) containing 12,000 cpm was added to the assay, and 200 µl of R48 was added to all tubes except the Totals and NSBs. After vortex mixing, the assay was incubated at room temperature for 2 to 3-hours. A 1-in-40 dilution of DARS was prepared in PBS-Gel containing 10% (w/v) of ethylenediamine tetra-acetic acid (EDTA, disodium salt, Fisons) in 0.15 M PBS. Donkey anti-rabbit serum and NRS at 1-in-400 in PBS-

Gel were dispensed in 100 µl volumes into all assay tubes excluding the Totals. The assay was vortex-mixed and incubated overnight at 4°C. The centrifuge (DPR-6000 or PR-7000; Du Pont) was pre-cooled to 4°C and immediately before spinning the assay, 1 ml of ice-cold PBS-Gel was added to all tubes except the Totals. Following centrifugation at 800 x g, 4°C for 30-minutes, the assay tubes were immediately inverted and the supernatents decanted to waste. The tubes were drained for 15-minutes on tissue paper before counting the pellets on a 60-second gamma-counter program and the data collected on AssayZap™.

TABLE 2.3: Procedure for the radioimmunoassay of 17ß-oestradiol.

TUBE	DAY 1							DAY 2
	Standard (µl)	PBS-Gel (µl)	Label (µl)	Antibody (µl)		NRS (µl)	DARS (µl)	
Totals	NIL	NIL						
NSB	NIL	700						
Zero Std	NIL	500						
0.5 pg/ml	5	495	50 µl	50 µl to	vortex	50 µl	50 µl	Centrifuge
0.75 pg/ml	7.5	492.5	to all	all tubes	mix, 2-h,	to all	to all	all tubes
1.0 pg/ml	10	490	tubes	*	room temp	tubes	tubes	(except
2.0 pg/ml	20	480			incubation	**	**	Totals) 800 x
3.0 pg/ml	30	470						g, 4°C, 30-
4.0 pg/ml	40	460						min. Decant
6.0 pg/ml	60	440						supernatents
8.0 pg/ml	80	420						and count
12.0 pg/ml	120	380						pellets for
16.0 pg/ml	160	340						60-s on
24.0 pg/ml	240	260						gamma-
48.0 pg/ml	480	20						counter
Control 1	500	NIL						
Control 2	500	NIL						
Control 3	500	NIL						
Recovery Label	10 µl of ^{3}H-oestradiol	490						
Unknown 1 Unknown 2 etc	Appropriate dilutions of samples in 500 µl							

* except Total and NSB tubes
** except Totals

2.1.6 Progesterone radioimmunoassay

Standard concentrations of progesterone (SIGMA) were prepared at 10, 20, 40, 60, 100, 150, 200, 250, 400, 500 and 750 pg/tube, to which 50 µl of serum from long-term castrated cockerels was added, and the final volume adjusted to 500 µl with PBS-Gel. The plasma samples were diluted in PBS-Gel to a volume of 500 µl.

All tubes received 10,000 cpm of ^{125}I-progesterone (iodinated by the method of Corrie *et al.*, 1982; supplied by MRC Reproductive Biology Unit, Edinburgh) in 100 µl PBS-Gel containing 1 mg/ml 8-anilo-1-naphthalene sulphonic acid (ANS; SIGMA) and, with the exception of the Totals and NSB tubes (containing 500 µl PBS-Gel only), also received 200 µl of 1-in-8000 rabbit anti-progesterone (R31/8) in PBS-Gel. The reaction tubes were vortex-mixed and incubated at room temperature for 3-hours. The completion of the assay procedure was identical to that used for the 17ß-oestradiol radioimmunoassay, but that DARS and NRS were prepared at dilutions of 1-in-35 and 1-in-300 respectively.

2.2 GnRH Receptor Assays

2.2.1 Assay procedure

The assessment of chicken pituitary GnRH receptors was by the method of Bramley, Menzies and Baird (1985). Four GnRH ligands were iodinated (Bramley *et al.*, 1985) and evaluated for their binding potential to chicken pituitary glands; namely mammalian GnRH (mGnRH; Peninsula), chicken GnRH-II (GnRH-II; Peninsula), Buserelin ([D-Ser(Bu^t)^6-desGly^{10}-NH$_2$]-GnRH-N-ethylamide); a gift to TA Bramley from J Sandow, Hoechst Pharmaceuticals) and D-Trp^6-GnRH-ethylamide (D-Trp^6 EtAmide; SIGMA).

Pituitary glands were frozen on dry ice and stored at -80°C until assayed for LH. The pituitary glands were thawed and homogenised (15 strokes by Dounce homogeniser) in 5 ml ice-cold SET buffer (0.3 M sucrose, 1 mM EDTA, 10 mM TRIS-HCl, pH 7.4). The pituitary homogenate (10 - 100 µl) was incubated in triplicate tubes at 4°C in 0.5% (w/v) BSA-40 mM TRIS-HCl buffer (pH 7.4) with 100,000 cpm tracer in a 1 ml system. After 4-hours, 500 µl of 0.5% (w/v) bovine γ-globulin in 40 mM TRIS-HCl, pH 7.4 was added and immediately followed by 1 ml ice-cold 25% (w/v) polyethylene glycol (6000 molecular weight) in 40 mM TRIS-HCl, pH 7.4, vigorously mixed and the tubes centrifuged at 1500 x g, 4°C for 15-minutes. The supernatents were discarded and the pellets counted for ^{125}I on a gamma-counter. Non-specific binding was measured by including 10 µg unlabelled tracer in the incubation tubes. A value for specific binding was obtained by subtracting the non-specific binding counts from the bound counts of the sample.

In some experiments, protease and peptidase inhibitors were used; phenylmethyl sulphonyl fluoride (PMSF; SIGMA), EDTA, and N-tosyl-L-phenylalanine chloromethyl ketone (SIGMA).

2.2.2 Purification and iodination of D-Arg^6-GnRH-II

A GnRH receptor assay for chicken pituitary tissue was developed to measure receptor number and affinity for GnRH. The analogue D-Arg^6-GnRH-II, is highly resistant to metabolic degradation and is a potent releasing hormone of LH in the chicken *in vivo* (Millar *et al.*, 1986; Sharp *et al.*, 1986b).

pyro-Glu^1-His^2-Trp^3-Ser^4-His^5-**D-Arg^6**-Trp^7-Tyr^8-Pro^9-Gly^{10}-NH_2

The elution characteristics of D-Arg^6-GnRH-II (a gift from RP Millar) were examined by HPLC (Waters, Model 490E uv detector and 600E pump). All buffers were freshly prepared and passed through a 5 cm 0.22 µm GV-filter (Millipore) before use. The analogue was separated on a 30-minute linear gradient of 12 - 60% (v/v) acetonitrile (HPLC grade S, Rathburn Chemicals Ltd) in 40 mM ammonium acetate (Merck), pH 4.0, on a 250 mm x 4.6 mm ODS Spherosorb reverse phase chromatography column (Anachem Ltd) at 25°C with a flow rate of 1.5 ml/min. This information provided the conditions necessary for isocratic studies; 7-minute run time at 25°C with a flow rate of 1.5 ml/min of 35.4% (v/v) acetonitrile in 40 mM ammonium acetate (pH 4.0).

The iodination procedure was modified from Greenwood *et al.* (1963). Five µg of analogue in 10 µl water, 10 µl of 0.93 µg/ml sodium iodide (Fisons) and 10 µl of 50 mM sodium phosphate buffer (pH 7.5) were mixed in a borosilicate glass test tube and the reaction started by adding 10 µl of 1 mg/ml chloramine T. The mixture was vortex-mixed at approximately 15-second intervals and the reaction proceeded at 25°C. The reaction was terminated after 60-seconds with 10 µl of 2 mg/ml sodium metabisulphite and the mixture acidified with 50 µl 1.0 M acetic acid. The elution profile was monitored at 280, 230 and 210 nm. The effects of reaction time and analogue concentration on the yield of iodinated product were examined. In addition, a series of control runs identified the retention times of individual reagents and their combinations.

FIGURE 2.2 shows the separation trace for the iodination of the analogue. Calculation of the specific activity indicated that the product was mono-iodinated with a yield of 28.0 ± 0.1%.

This procedure was used to label the analogue with ^{125}I.

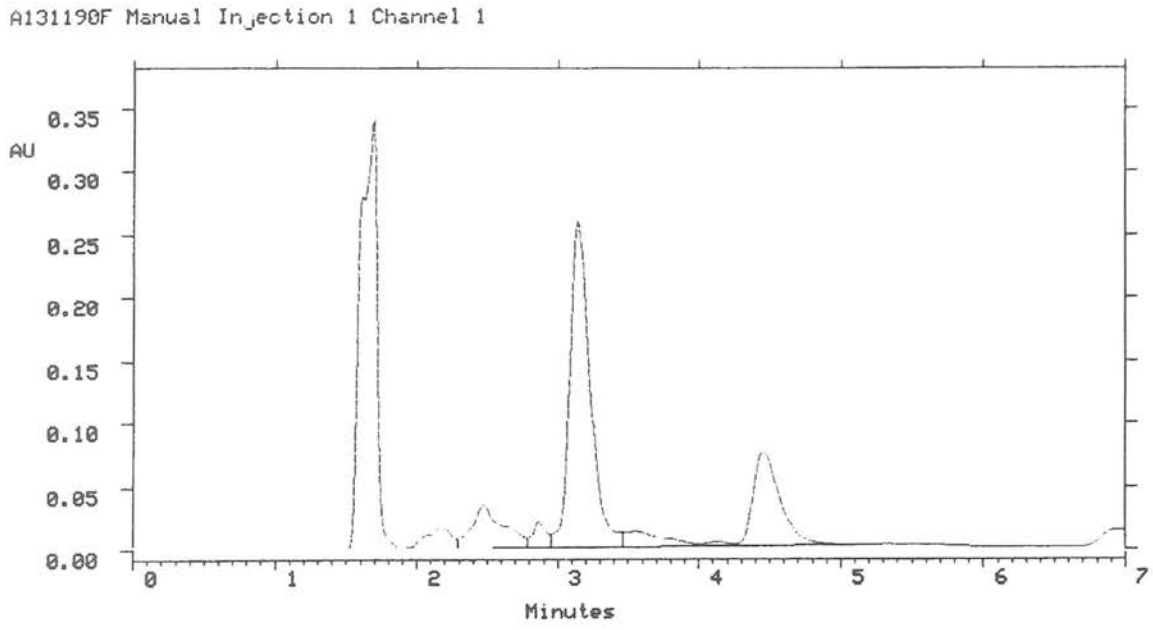


FIGURE 2.2: Separation profile by HPLC of the reaction mixture after the iodination of D-Arg^6-GnRH-II.

2.3 LH DETERMINATIONS ON PITUITARY EXPLANTS

2.3.1 Total pituitary LH

Pituitary glands were removed and weighed by torsion balance. The whole pituitary gland was homogenised manually using a Teflon pestle in a 1.5 ml microcentrifuge tube containing 500 µl of ice-cold 50 mM phosphate-buffered saline (PBS, pH 7.5). Following centrifugation at 13,000 x **g** (Biofuge A, Heraeus Sepatech) for 10-minutes at room temperature, the supernatent was frozen at -20°C for later LH radioimmunoassay.

2.3.2 'Readily releaseable' pool of LH

Pituitary glands were weighed and cut into quarters to increase surface area. The pituitary tissues were allocated to 18 mm diameter 24-well tissue culture plates (Costar Ltd) containing avian ringer (AR; see below). The pituitary tissue was maintained at 41°C in an humidified atmosphere of 5% CO_2 : 95% air with continuous orbital shaking at 0.5 Hz (Denley-Luckham Ltd). The tissue was washed by replacing the AR every 30-minutes for 3-hours (sufficient to establish a stable baseline in preliminary experiments). The pituitary gland was rinsed in fresh AR and the basal release of LH determined by a 30-minute incubation in AR. Following a further rinse in AR, the medium was replaced with a depolarising concentration of 60 mM KCl in AR (NaCl concentration reduced accordingly to maintain isotonicity) for 30-minutes. The samples were stored at -20°C until

assayed for LH. The 'readily releaseable' pool (RRP) of LH was calculated by subtracting the basal release of LH from the K^+-releaseable LH.

Avian Ringer

4 mM KCl	116 mM NaCl
2.2 mM $CaCl_2$	1 mM $MgSO_4.7H_2O$
0.41 mM $NaH_2PO_4.2H_2O$	29.2 mM $NaHCO_3$
0.2% (w/v) glucose	0.15% (w/v) bacitracin
0.1% (w/v) BSA	

pH adjusted to 7.4. The KCl concentration was increased to 60 mM, and the concentration of NaCl reduced to 60 mM in some experiments. In experiments using pituitary cell cultures, Buffer A replaced AR.

Buffer A

140 mM NaCl	4 mM KCl
1 mM $MgCl_2.6H_2O$	1.4 mM Na_2HPO_4
8.3 mM glucose	20 mM HEPES

The pH was adjusted to 7.4. The KCl concentration was increased to 60 mM, and the NaCl concentration reduced to 84 mM in some experiments.

2.4 STUDIES *IN VIVO*

2.4.1 Plasma half-life of ^{125}I-LH

Iodinated LH (^{125}I-LH; SECTION 2.1.4.1.1) was diluted 1-in-2 with saline and each bird received an intravenous dose of 0.78 MBq ^{125}I-LH in 100 µl. Timed bleeds were collected over 60-minutes. The blood was centrifuged and the plasma diluted 1-in-2 with 100 µl RIA diluent and equilibrated with 50 µl of 1-in-190 LH antiserum (antiLH-3/3) overnight at 4°C, followed by a further overnight incubation at 4°C with 50 µl of 1-in-4 donkey anti-rabbit serum (DARS) and 50 µl 1-in-200 normal rabbit serum. All samples were processed in duplicate. Triplicate NSB tubes contained 200 µl RIA diluent and were processed in parallel with the sample tubes. The precipitate was centrifuged at 1500 x **g**, 4°C for 30-minutes and 50 µl of 6% (w/v) starch suspension added. After centrifuging the tubes for a further 15-minutes the supernatents were discarded and the ^{125}I activity in the pellets counted for 60-seconds per tube. The NSB counts were subtracted from all sample counts, and these specific counts were plotted against time after injection.

2.4.2 Passive immunisation against GnRH-I

Laying hens which had an uninterrupted sequence of at least 8 eggs, were used in these studies. Following a pre-dose bleed, either 2 ml sheep anti-chicken GnRH-I serum or 2 ml normal sheep serum was intravenously injected into cockerels between 10:00 - 10:30-h, and 13:00 - 14:00-h in laying hens. Blood samples were collected at 0, 0.5, 2, 4, 6 (only in cockerels) and 24-hours after injection. Egg-laying in the hen was checked daily for 3 days. Anti-chicken GnRH-I serum was prepared by the method of Sharp *et al.* (1990) using sheep instead of rabbits.

In a second experiment, laying hens were entrained to a 28-hour light cycle (14L:14D) to synchronise the preovulatory LH surge (Morris *et al.*, 1975). Blood samples were collected at 2-hour intervals and 2 ml of normal sheep serum or anti-GnRH-I serum injected at 4-hour intervals between 13:00-h (lights off) and 23:00-h. Again egg-laying was checked daily for 3 days after treatment.

The binding activity of the anti-GnRH-I serum was measured in the plasma samples. Firstly, an optimum dilution of plasma was identified by processing serial dilutions of plasma in the assay. Two hundred µl of diluted plasma (in RIA diluent) was then incubated overnight at 4°C with 50 µl ^{125}I-GnRH-I (12,000 cpm/50 µl). Non-specific binding (NSB) tubes contained 200 µl RIA diluent and 50 µl label, while the 'Totals' contained only label. On Day 2, 50 µl of both donkey anti-sheep serum (1-in-20; Scottish Antibody Production Unit) and normal sheep serum (1-in-200; Scottish Antibody Production Unit) were added to the assay tubes (except 'Totals'). After a further overnight incubation at 4°C, the tubes (except 'Totals') were centrifuged at 1500 x **g**, 4°C for 30-minutes and 50 µl of 6% (w/v) starch suspension added. The tubes were centrifuged for a further 15-minutes. The supernatents were discarded and ^{125}I activity in the pellets counted for 60-seconds per tube. The fraction of ^{125}I-GnRH-I specifically bound to the plasma sample was calculated by subtracting the NSB counts from the sample counts and expressing as a percentage of the 'Total' counts.

The theoretical amount of ^{125}I-GnRH-I that could be bound by 1 ml of plasma was calculated by using the specific activity of 1620 µCi/µg for ^{125}I-mammalian GnRH (Bolton, 1977). A sample calculation is given below.

Total counts per tube = 9010 cpm

Counts in 200 µl plasma diluted 1-in-200 = 2814 cpm

Gamma-counter efficiency = 75%

2814 cpm + [2814 cpm x (75/100)] = **3752 dpm bound**

Specific activity for ^{125}I-mammalian GnRH (Bolton, 1977) = 1620 µCi/1 µg

1 µg $\equiv$ 1620 µCi

Since $1\ \mu Ci = 2.2 \times 10^6$ dpm

$\therefore$ $1\ \mu g \equiv 3.564 \times 10^9$ dpm

$\therefore$ $1\ pg \equiv 3564$ dpm

$$\frac{3564 \text{ dpm}}{1 \text{ pg}} \equiv \frac{3752 \text{ dpm}}{\text{UNK pg}}$$

$\therefore$ UNK = 1.053 pg

200 µl of 1-in-200 diluted plasma

$\therefore$ 210.5 pg/200 µl neat plasma $\equiv$ **1053 ng GnRH-I per ml of plasma**

The effect of the anti-GnRH-I treatment on the preovulatory surge of LH was tested by analysis of variance of the $\log_{10}$-transformed values of LH (StatView-II).

2.5 Processing of Chicken Pituitary Glands for Transmission Electron Microscopy

The anterior pituitary gland was removed within 1.5-minutes of death, longitudinally bisected and fixed for 1-hour at 4°C in 1% (w/v) osmium tetroxide in modified Dalton's buffer (Rothwell, 1978). The fixed material was dehydrated through ethanol; 5 and 10-minutes in 50%, 2 x 20-minutes in 95% and 2 x 25 in alcohol; cleared by two 5-minute washes in Inhibisol (Penetone Ltd; Maxwell, 1978) and incubated overnight at room temperature in equal parts of Inhibisol and Araldite (CY212, TAAB Labs). The tissue was incubated for a further 48-hours in fresh Araldite, embedded and cured over 48-hours at 60°C. Ultrathin 'gold-silver' sections were cut (Ultrotome III, LKB) and mounted onto 200-mesh copper grids (Agar Scientific Ltd). The sections were stained with 2% uranyl acetate in 50% ethyl alcohol for 2-minutes (Watson, 1958) and lead citrate (Reynolds, 1963) for 5 - 7-minutes, washed twice in distilled water, dried on filter paper and examined in an electron microscope (Philips EM 300).

Osmium tetroxide in modified Dalton's buffer

3% (w/v) potassium dichromate (Merck), pH to 7.0 with KOH
2.6% (w/v) sodium chloride (Merck)
2% (w/v) osmium (VIII) tetroxide (Johnson Matthey Materials Technology)

Dalton's buffer was prepared with 100 ml potassium dichromate and 100 ml sodium chloride and the pH adjusted to 7.0 with KOH. Equal parts of Dalton's buffer to osmium tetroxide were combined immediately before use and the osmolarity adjusted to 330 mOs.

2.5.1 Quantitative image analysis

The cell-types of the chicken anterior pituitary gland were identified ultrastructurally by the classification of Tai and Chadwick (1977) and Tai (1976). Full size pictures were taken of gonadotroph cells which included a nuclear profile. The electron microscope negatives (100 x 80 mm) were illuminated on a light-box, viewed by video camera and analysed by a Quantimet 570 image analyser (Cambridge Instruments).

A program was written to measure the distribution of secretory granules in gonadotroph cells. Basically, a line was fitted to each cell, which was equidistant from the cell membrane and the nucleus, such that the cytoplasmic area was divided into an inner (perinuclear) and an outer (subplasmalemmal) region. The equipment was calibrated and used to measure the number and the size of the secretory granules within the two regions, and also to measure the areas of the cytoplasmic regions and the nucleus.

2.5.2 Acid phosphatase

Pituitary tissue was processed for the identification of acid phosphatase at the ultrastructural level by the method of Barka and Anderson (1962).

An incubation solution of 10 ml 0.2 M TRIS-maleate buffer (Tris[hydroxymethyl]amino-methane; Merck, pH 5.0) with 10 ml water and 10 ml of freshly prepared 1.25% (w/v) sodium ß-glycerophosphate (Merck; adjusted to pH 5.0 with 1 M HCl) was mixed continuously, to which 20 ml of 0.2% (w/v) lead nitrate in distilled water was added by dropper. Ultrathin sections were incubated in this solution for 30-minutes at 37°C with constant agitation. Subsequent to rinses in distilled water, the sections were lightly stained with lead citrate, air-dried and then visualised by electron microscopy.

2.5.3 Potassium ferricyanide

Fresh pituitary glands were bisected longitudinally and fixed for 16-hours at 4°C in a solution of 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate/HCl buffer in distilled water (pH 7.4) containing 0.05 M $K_3Fe(CN)_6$ (Merck; De Bruijn & Den Breejen, 1976). The fixed tissue was processed through ethanol and through all succeeding steps as described above, except that the sections were stained with lead citrate for only 5-minutes.

2.5.4 Lipid-staining of pituitary cryostat sections

Pituitary glands were collected into a mixture of *iso*-pentane/dry ice, mounted in OCT Compound (BDH Chemicals Ltd) from which 10 µm frozen sections were cut using a Brights cryostat (Cambridge rocking-type). All sections were thaw-mounted onto glass slides to which a drop of 10% (v/v) ethanol was added. The excess liquid was removed and the sections incubated overnight in a sealed and darkened jar with 2% (w/v) osmium tetroxide in distilled water. After rinsing in distilled water, the sections were lightly stained with haematoxylin for 30-seconds, and then

immersed into ammonia fumes for 2-seconds and followed by a further rinse in water. The sections were mounted in glycerol/PBS and examined by light microscopy.

2.6 STUDIES *IN VITRO*

2.6.1 Static incubations of pituitary tissue

Hemipituitary glands were allocated randomly to individual wells of a 24-well plate (Costar Ltd). The tissues were equilibrated in 6 x 30-minute washes of 500 µl 25 mM HEPES- (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], SIGMA) or 26.2 mM sodium bicarbonate-buffered Medium 199 (M199; SIGMA) or Dulbecco's modified Eagle's medium (DMEM; SIGMA) each containing 2% (v/v) steroid-stripped newborn calf serum (NBCS, SIGMA; see below) at 37°C in an humidified atmosphere of 5% CO_2 : 95% air, and gently agitated by orbital shaker (Denley-Luckham Ltd). The tissues were rinsed with fresh medium before replacing with GnRH-I-containing medium. Pituitary tissues were weighed after each experiment.

Steroids were removed from NBCS and foetal calf serum (FCS; Flow Labs) by mixing overnight at 4°C with 1% (w/v) activated charcoal (Merck) and 0.1% (w/v) dextran T-70 (Pharmacia) followed by centrifugation at 20,000 x g at 4°C for 30-minutes (Beckman J2-21M/E, JA-20 rotor). Finally, the serum was sterilised through a 0.22 µm membrane filter (Costar Ltd).

In time-course experiments, a protocol was used which ensured that each treatment group received the same total period in culture (FIGURE 2.3). After the wash procedure, the hemipituitary glands for the 150-minute time-point were rinsed in 500 µl medium and then replaced with 500 µl fresh medium in the presence and absence of GnRH-I. As the experiment proceeded, hemipituitary glands for the 90-minute time-point were similarly treated and so on such that all samples of medium were retrieved at 150-minutes and frozen at -20°C for later LH radioimmunoassay. Pituitary weights were measured at the end of the experiments.

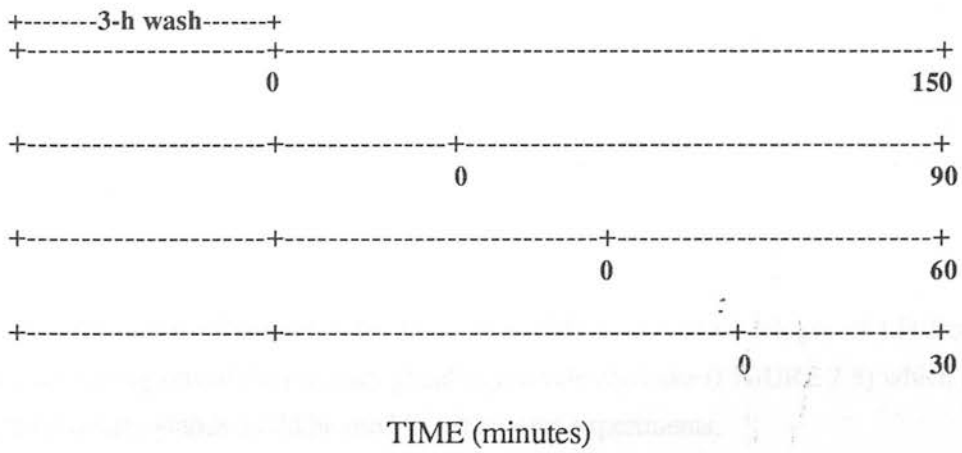


FIGURE 2.3: Protocol for measuring the time-course of GnRH-I-stimulated LH release.
Individual hemipituitary glands from chickens of mixed sex were washed in M199-NBCS. After 3-h, the tissue was rinsed and the medium replaced with fresh M199-NBCS ± GnRH-I. The changes of medium were timed such that all tissues received the same total period in culture.

When 4 combinations of culture medium and buffer system were compared, there were no differences between the basal concentrations of LH, however GnRH-I stimulated a smaller release of LH ($P<0.01$) in HEPES-buffered DMEM compared with other culture media (FIGURE 2.4). The time-course of LH release from GnRH-I-stimulated pituitary pieces maintained in bicarbonate-buffered M199 is shown in FIGURE 2.4. A 60-minute incubation was chosen for later studies because this time-point lay on the linear portion of the time-course curve of LH release by GnRH-I.

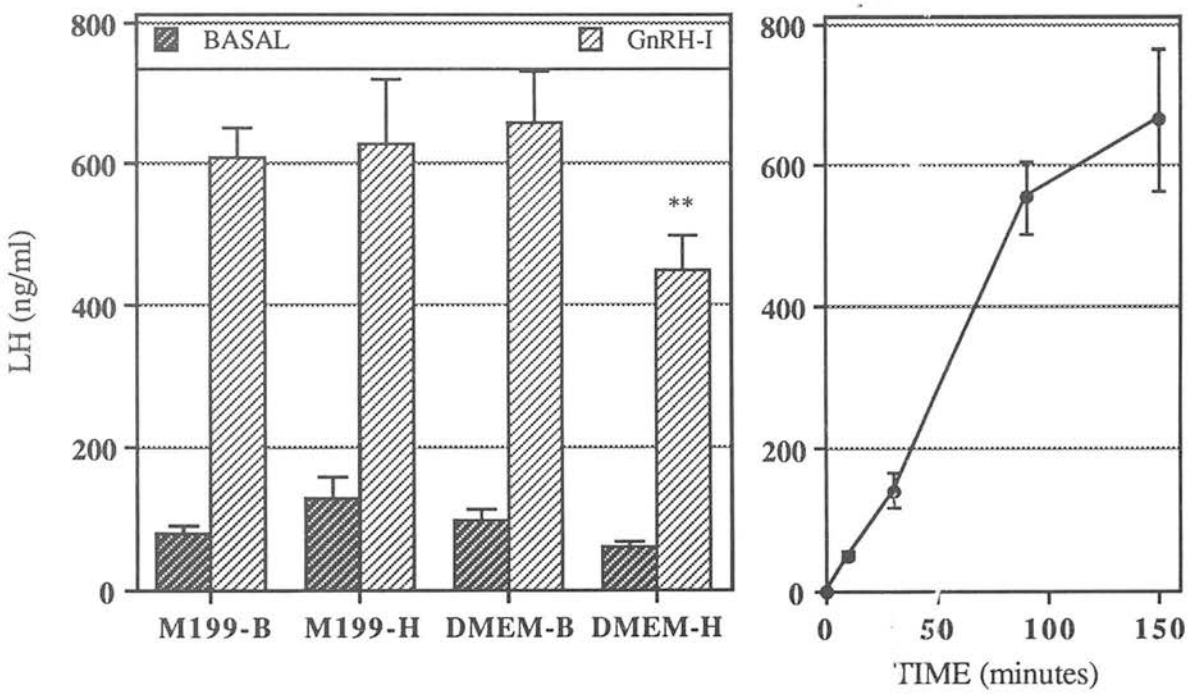


FIGURE 2.4: Comparison of the effect of culture medium and buffer on the basal and GnRH-I-stimulated release of LH from hemipituitary glands from juvenile chickens. Hemipituitary glands (mixed sexes; n = 8 per treatment) were washed and equilibrated for 3-h with HEPES- (H) or bicarbonate- (B) buffered M199 or DMEM before incubating with or without 100 nM GnRH-I for 90-min prepared in the same combination of medium and buffer (left-hand panel). **$P<0.01$ compared with the GnRH-I-stimulated LH concentration from pituitary tissue maintained in all other media. The time-course of LH release in M199-B from hemipituitary glands by 100 nM GnRH-I (basal LH subtracted) is shown in the right-hand panel.

There was no significant difference in the basal or GnRH-I-stimulated release of LH from the cephalic and caudal regions of the pituitary gland of juvenile chickens (FIGURE 2.5) which meant that quartered pituitary glands could be used in subsequent experiments.

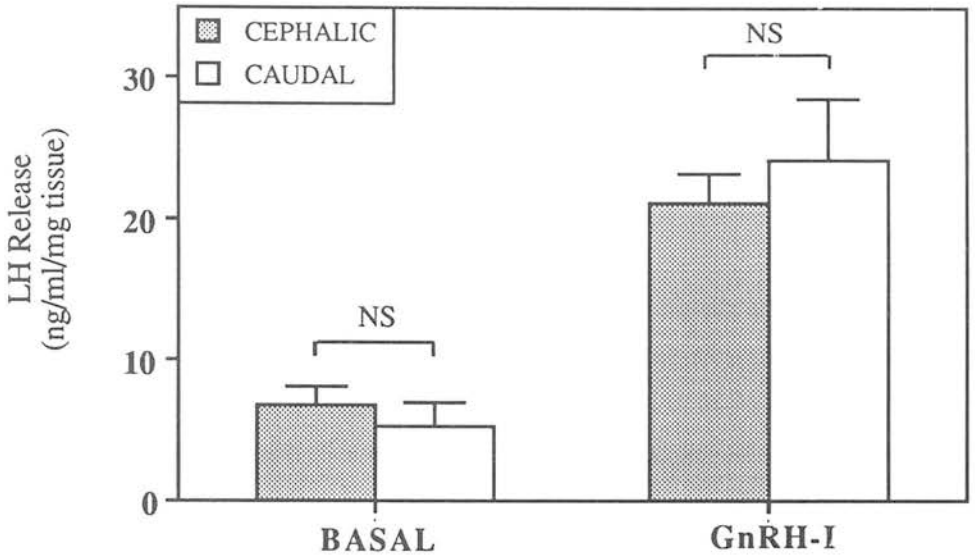


FIGURE 2.5: Comparison of LH release from cephalic and caudal lobes of pituitary glands from juvenile chickens.
LH release was measured from portions of cephalic and caudal lobe of pituitary glands from juvenile chickens (intermediate region discarded; mixed sexes) ± 10 nM GnRH-I for 60-min. NS = not significantly different, n = 8.

2.6.2 Perifusion of pituitary tissue

Pituitary glands were either quartered, or mechanically chopped (Tissue Chopper, Mickle Laboratory Engineering Co.) into 300 µm slices, rinsed in perifusion medium (see below) and placed in 0.4 ml chambers (made from 1 ml Plastipak syringes; Becton Dickinson) containing 100 µl pre-swollen P-2 Bio-Gel (BIO-RAD) supported on a disc of GF/C glass fibre filter (Whatman; cut with # 2 cork borer). Each chamber was sealed with its own plunger which was pierced with stainless steel tubing (1 mm internal diameter) secured in position with a welded stainless steel collar (IAPGR, Dept. of Engineering Services). The internal chamber volume was adjusted to 200 µl and the pituitary tissue washed for 3-hours by constant perifusion (Gilson Minipuls-2 peristaltic pump located between the chambers and the Ultrorac 7000 fraction collector, LKB-Pharmacia; white-orange tubing, i.d. 0.64 mm; Gradko) at 250 µl/min with perifusion medium (see below) at 41°C (Techne Circulator C-100) gassed with 5% CO_2 : 95% O_2. All air bubbles were expelled from the apparatus at the start of the wash period and their absence was monitored at intervals throughout each experiment. In preliminary studies, the flow rate was periodically checked throughout the experiment and found to be 255.8 ± 6.2 µl/min (mean ± sem). In later experiments, only the perifusion rates at the start and end were checked. The flow rate characteristics of the system were determined by measuring the absorbance (280 nm) of 1-minute perifusate fractions after introducing a 4-minute pulse of 0.1% (w/v) bovine serum albumin (RIA grade; SIGMA) into the perifusion apparatus. During the experiments, 1 or 2-minute samples were retrieved at timed intervals and frozen at -20°C for later LH radioimmunoassay. The wet pituitary weight was measured at the end of each experiment.

2.6.2.1 Validation of the perifusion apparatus

The flow rate characteristics for each channel of the perifusion apparatus is shown in FIGURE 2.6. The within-channel and between-channel fluctuations in pump volume per fraction were 4.8% and 7.2% respectively.

The choice of perifusion medium depended on the experiment. Bicarbonate-buffered, phenol red-free M199 containing 2% (v/v) steroid-stripped NBCS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (SIGMA) was used in all experiments, with the exception of the high K^+ depolarisation studies where avian ringer and 60 mM K^+-avian ringer were used.

In experiments which investigated the role of calcium ions on GnRH-I-stimulated LH secretion, M199-NBCS was replaced by phenol red-free Earle's balanced salt solution (EBSS, GIBCO) or calcium- and magnesium-free EBSS containing penicillin and streptomycin, and 2% (v/v) steroid-stripped NBCS (EBSS-NBCS). Magnesium chloride was added to the calcium- and magnesium-deficient EBSS to restore the normal concentration and 1.5 mM EGTA was added. A 10 mM stock solution of the dihydropyridine nifedipine (NFP; SIGMA) was freshly prepared in ethanol, and dissolved in normal EBSS to give a final concentration of 20 µM NFP (final ethanol concentration of 0.2%).

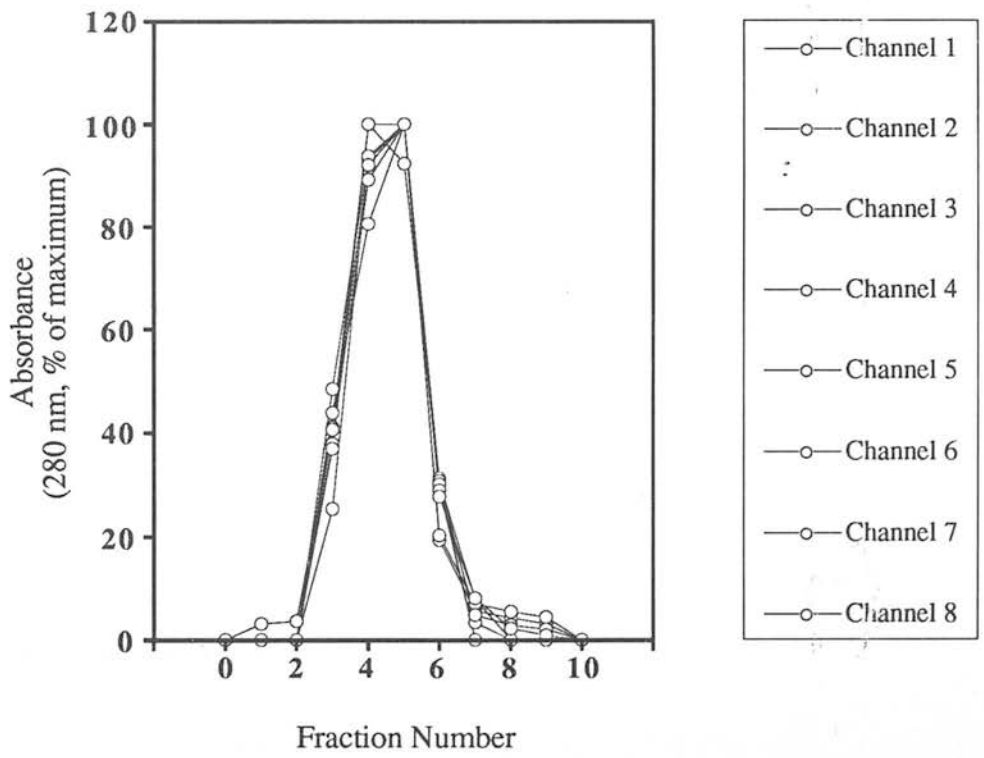


FIGURE 2.6: The flow characteristics of the perifusion apparatus.
The perifusion apparatus was infused with water (41°C) at 250 µl/min followed by a 4-min pulse of 0.1% BSA. One-min fractions were collected and the absorbance measured at 280 nm and expressed as a percentage of maximum absorbance.

2.6.3 Pituitary cell cultures

2.6.3.1 Preparation of dispersed pituitary cell cultures

In preliminary experiments, 25 mM HEPES or 26.2 mM sodium bicarbonate-buffered M199 or DMEM were compared to find the optimum combination to support chicken gonadotroph cells in culture. Anterior pituitary glands were collected into sterile bicarbonate-buffered M199 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 2% (v/v) steroid-stripped NBCS (all reagents from SIGMA). All subsequent procedures were performed in a laminar air cabinet (Flow Labs) using aseptic technique, and sterile solutions and equipment. After washing with 4 x 10 ml fresh M199-NBCS, the pituitary glands were minced with a 10a scalpel blade (Swann-Morton). The tissue was incubated in Dissociation buffer (see below) containing 1400 U/ml collagenase (Type I; Worthington Biochemicals Corp) and 40 U/ml deoxyribonuclease I (SIGMA) at 37°C in a water bath shaking at 0.5 Hz. At 10-minute intervals the dispersion of pituitary tissue was facilitated by gentle pasteur pipette action. Typically the dispersion procedure lasted 40 - 50-minutes. The suspension was centrifuged at 200 x **g** for 5-minutes at room temperature and the pellet was resuspended in EGTA-BSA buffer (140 mM NaCl, 4 mM KCl, 1.4 mM Na_2HPO_4, 8.3 mM glucose, 20 mM HEPES, 0.5 mM EGTA, 1% (w/v) BSA, pH 7.4). This centrifugation step was repeated, followed by a second resuspension in EGTA-BSA buffer. The cells were filtered through a 64 µm nylon mesh (Lockertex) mounted in a Swinnex holder (Millipore) and the filtrate centrifuged once more and resuspended in M199 containing 10% (v/v) steroid-stripped foetal calf serum (FCS; GIBCO). The cells were counted by haemocytometer and the viability of the cells assessed using 0.4% trypan blue (SIGMA).

Dissociation buffer

137 mM NaCl	5 mM KCl
0.7 mM Na_2PO_4	25 mM HEPES
0.36 mM $CaCl_2.2H_2O$	10 mM glucose
1% (w/v) BSA	

The pH was adjusted to 7.2.

A similar procedure for the dissociation of pituitary glands by 0.2% (w/v) trypsin (Flow Labs) was also followed, except that bicarbonate-buffered M199-NBCS replaced Dissociation buffer and EGTA-BSA buffer.

The final suspension of cells in M199-FCS was dispensed into tissue culture-treated 35 mm Petri dishes, or 24 or 48-well plates (all from Costar Ltd) and cultured at 37 or 41°C in an humidified atmosphere of 5% CO_2 : 95% air.

2.6.4 Optimisation of pituitary dispersion and cell culture conditions

2.6.4.1 Choice of dispersion procedure - trypsin versus collagenase

The two most commonly used enzymes for dispersing chicken pituitary tissue are collagenase (Millar & King, 1983; Perez *et al.*, 1989) and trypsin (Vasilatos-Younken, 1986; Wilson *et al.*, 1990a). The methods were compared for their suitability in obtaining a viable preparation of cells for LH secretion studies.

The trypsin protocol yielded fewer cells per pituitary gland from juvenile chickens (1.04 ± 0.13 million, n = 15) with a lower cell viability (87.7 ± 1.0%, n = 15) compared with the collagenase-based method which yielded 1.82 ± 0.22 million cells per pituitary gland ($P<0.001$, n = 25) and a cell viability of 97.5 ± 0.7% ($P<0.001$, n = 25). The trypsin method produced a suspension of single cells, whereas a preparation of mainly single cells and some undissociated aggregates of 2 - 4 cells was obtained with collagenase. Both cell preparations attached to plastic culture dishes within 3-hours. A variable but low proportion (< 5%) of red blood cells was present using both dispersion techniques however no attempt was made to remove them in these studies.

GnRH-I stimulated the release of LH from freshly dispersed (collagenase) pituitary cells when newborn calf serum (NBCS) was included in the stimulation medium (FIGURE 2.7), but not when the cells were incubated in NBCS-deficient medium. A protein supplement of 2% NBCS (containing 5.5 - 6.5 g protein/100 ml NBCS; SIGMA) or 0.1% BSA was therefore added to the medium in all stimulation studies.

Increasing concentrations of GnRH-I also increased the release of LH from freshly dispersed cell suspensions prepared using trypsin ($P<0.05$ at all concentrations of GnRH-I, compared with basal LH, n = 8). However, the incremental changes in LH were small and the procedure was associated with a high background concentration of LH in cells cultured for up to 72-hours (FIGURE 2.8).

Except where otherwise indicated, collagenase was used to disperse pituitary tissue for the LH secretion studies.

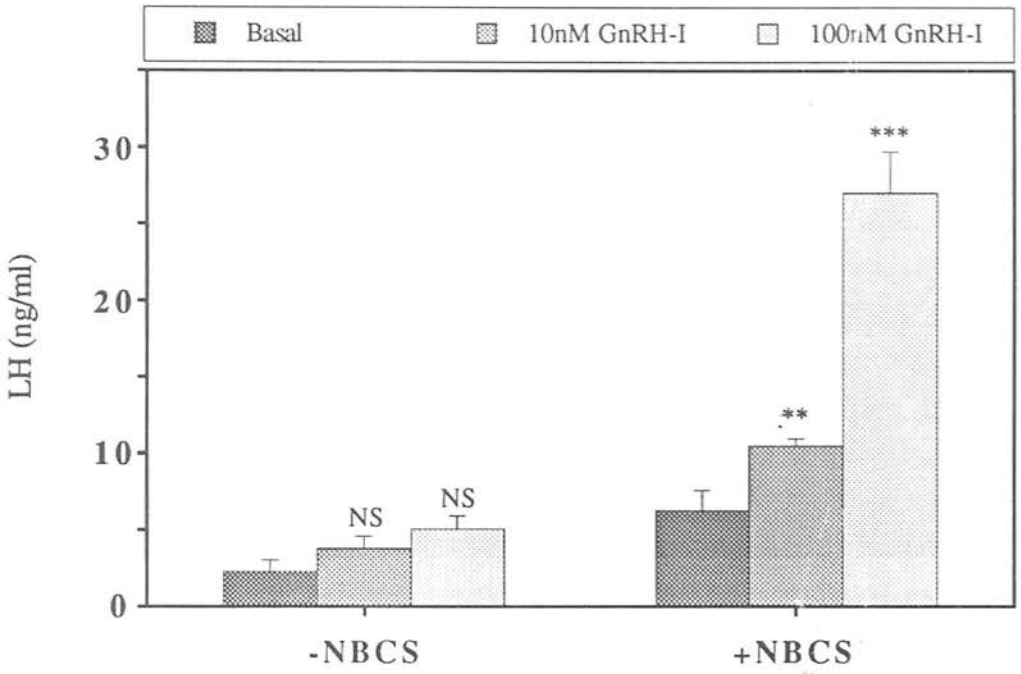


FIGURE 2.7: Effect of newborn calf serum (NBCS) on the GnRH-I-stimulated release of LH from pituitary cells.
Pituitary glands from juvenile chickens were dissociated with collagenase and the cells dispensed into chilled (4°C) plastic test-tubes at a density of 500,000 per 250 µl Buffer A ± 2% NBCS (Buffer A-NBCS). A further 250 µl Buffer A ± GnRH-I in the presence or absence of NBCS was incubated with the cells at 41°C. After 60-min, the cells were centrifuged at 4°C and the supernatent assayed for LH. NS = not significantly different, **P<0.01, ***P<0.001 compared with respective basal LH (n = 8).

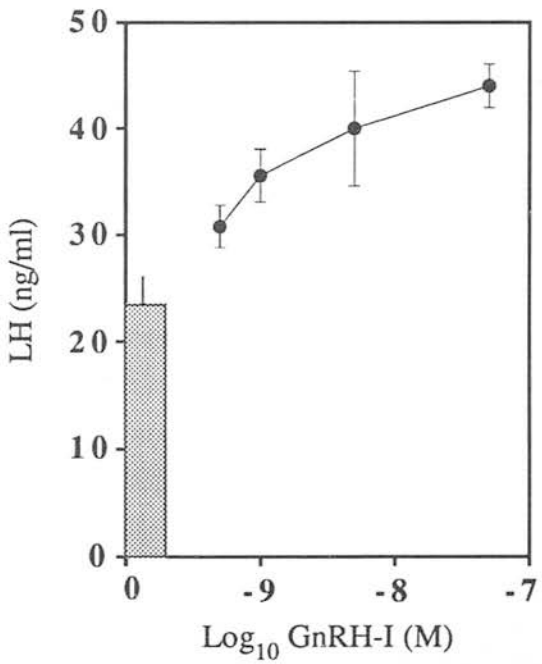


FIGURE 2.8: Dose-response relationship between GnRH-I and LH release from trypsin-dispersed cells.
Pituitary glands from juvenile chickens were dispersed with trypsin. Cells were stimulated with GnRH-I in Buffer A-NBCS (n = 8 per dose of GnRH-I).

2.6.4.2 Conditions for pituitary cell culture

2.6.4.2.1 Selection of serum supplement

Pituitary cells maintained in Medium 199 (M199) supplemented with foetal calf serum (FCS) had a lower unstimulated release of LH at all serum concentrations tested ($P<0.001$; TABLE 2.4) compared with chicken serum (CS). In addition, GnRH-I stimulated a greater release of LH (ΔLH) from FCS-incubated cells than from those incubated with CS ($P<0.001$ at all serum concentrations). The mean fold-increase above basal in LH secretion by GnRH-I (of the 3 serum concentrations) was 1.67 and 1.14 for FCS and CS respectively. Ten percent FCS was included in all subsequent cell culture media.

TABLE 2.4: Comparison between supplements of chicken and foetal calf serum on the basal and GnRH-I-stimulated release of LH from cultured pituitary cells.

		Serum Concentration		
		5%	10%	20%
Chicken	Basal	48.9 ± 2.3	46.6 ± 2.0	51.9 ± 2.4
	GnRH-I	$56.2 \pm 2.5^{*}$	$55.4 \pm 1.3^{***}$	$55\,9 \pm 1.7^{NS}$
Foetal Calf	Basal	33.9 ± 1.1	37.1 ± 1.7	$39.8 \pm 1.6^{\dagger}$
	GnRH-I	$59.4 \pm 0.9^{***}$	$61.6 \pm 0.9^{***}$	$62.1 \pm 0.9^{***}$

Collagenase-dissociated pituitary cells from juvenile chickens were cultured with chicken or foetal calf serum at 5, 10 or 20% in bicarbonate-buffered M199. After 48-h, the cells were rinsed and then replaced with Buffer A containing 0.1% (w/v) BSA ± 100 nM GnRH-I for 60-min (n = 8). NS = not significantly different, *P<0.05, ***P<0.001 compared with respective basal (within serum type).

Cells which were cultured in medium supplemented with steroid-stripped FCS released a lower baseline output of LH ($P<0.05$), were less responsive to GnRH-I stimulation ($P<0.05$ at 1 and 100 nM GnRH-I) than those maintained in untreated FCS (FIGURE 2.9), and the 2-point dose-response curves appeared to be parallel.

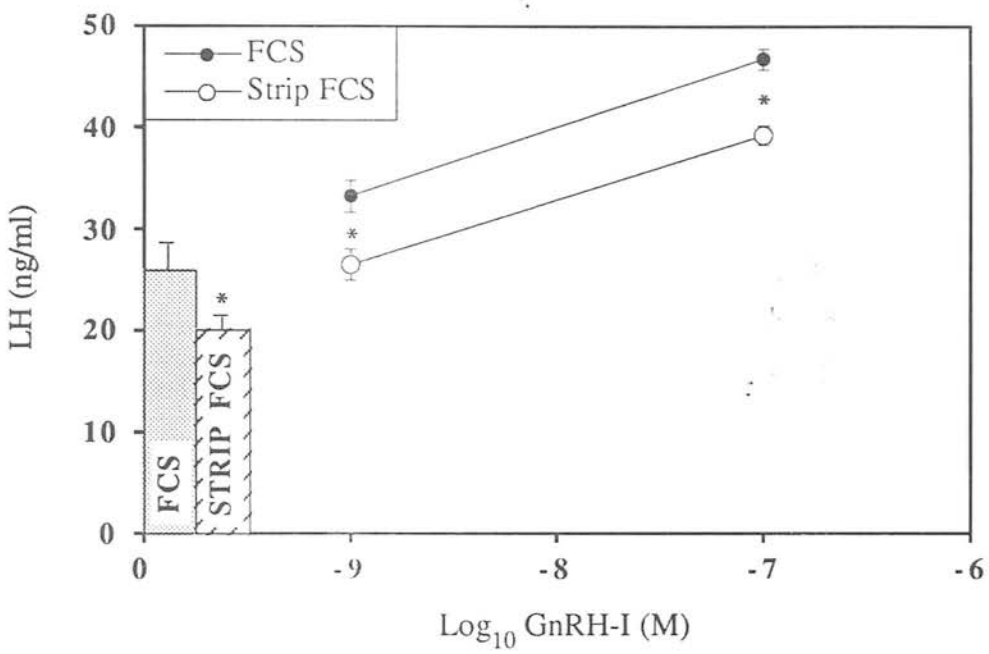


FIGURE 2.9: Effect of charcoal-treating foetal calf serum on the GnRH-I-stimulated release of LH.
Pituitary cells (500,000/500 µl) from juvenile chickens were cultured with charcoal-treated or untreated 10% FCS in bicarbonate-buffered M199. After 48-h, the cells were rinsed and then incubated for 60-min with Buffer A-BSA (0.1% w/v) ± 1 or 100 nM GnRH-I. *P<0.05 compared with untreated FCS, n = 8.

2.6.4.2.2 Selection of culture medium and buffer

Dulbecco's modified Eagle's medium (DMEM) and Medium 199 (M199) were originally developed for studying chicken cells *in vitro* (Freshney, 1987). Both media have been used to culture chicken pituitary cells (Luck & Scanes, 1980; Hasegawa *et al.*, 1984; Chou *et al.*, 1985; King *et al.*, 1986; Vasilatos-Younken, 1986; Perez *et al.*, 1989; Johnson & Tilly, 1991). Bicarbonate or HEPES buffer systems were also compared for their ability to maintain chicken pituitary cells.

GnRH-I increased the release of LH (P<0.05 compared with basal) from cells sustained in all combinations of medium and buffer at all culture periods with the exception of those incubated with DMEM after 48-hours of culture (not significantly different from basal). Similar concentrations of basal and GnRH-I-stimulated LH were seen in fresh cells when compared across media within the same buffer type (TABLE 2.5).

TABLE 2.5: Comparison of the effects of culture medium, buffer and culture period on the pituitary cell basal and GnRH-I-stimulated release of LH.

		FRESH	24-h	48-h
M199-B	Basal	12.2 ± 0.7	14.2 ± 1.2	23.3 ± 1.7
	GnRH-I	25.3 ± 1.1	28.5 ± 2.0	38.0 ± 1.4
		[2.1]	[2.0]	[1.6]
M199-H	Basal	18.5 ± 1.6	17.4 ± 1.0	22.5 ± 1.6
	GnRH-I	24.5 ± 1.3	25.4 ± 1.3	27.8 ± 1.1
		[1.3]	[1.5]	[1.2]
DMEM-B	Basal	11.6 ± 2.4	7.8 ± 0.9	10.8 ± 0.8
	GnRH-I	27.0 ± 2.5	12.5 ± 0.5	12.1 ± 1.7
		[2.3]	[1.6]	[1.1]
DMEM-H	Basal	16.6 ± 2.3	4.6 ± 0.6	7.3 ± 1.2
	GnRH-I	23.6 ± 2.1	9.6 ± 1.0	8.9 ± 0.4
		[1.4]	[2.1]	[1.2]

Pituitary cells from juvenile chickens were dispensed into test-tubes or culture wells (500,000 cells/500 µl M199-B-FCS). The cells in the test-tubes were washed once by centrifugation and replacing with fresh medium (500 µl bicarbonate or HEPES-buffered M199 or DMEM), and then incubated for 30-min at 41°C. The procedure for stimulating the cells with 100 nM GnRH-I is described in FIGURE 2.7. After a 5-h incubation in the wells, the medium was replaced with 500 µl bicarbonate or HEPES-buffered M199 or DMEM, and the cells cultured for 24-h or 48-h. Cells (n = 8) were incubated in a fresh volume of the same medium ± 100 nM GnRH-I for 60-min and the samples assayed for LH. The GnRH-I-stimulated fold-increase above the respective basal LH is given in square brackets.

The pH indicator phenol red (phenolsulphonphthalein) is frequently included in commercial cell culture media at a concentration of 20 - 60 µM (Freshney, 1987). However, phenol red is oestrogenic and can modify cell function and morphology in MCF-7 human breast cancer cells (Berthois *et al.*, 1986; Nelson *et al.*, 1987), and rat gonadotroph cells (Hubert *et al.*, 1986; Welshons *et al.*, 1988; Ortmann *et al.*, 1990). It was necessary to evaluate the effect of the pH indicator on chicken pituitary cell cultures because the action of steroids was to be studied in the present experiments.

In 48-hour pituitary cell cultures, the presence of 22.9 µM phenol red reduced the output of LH in unstimulated ($P<0.01$) and GnRH-I-stimulated cells ($P<0.001$) by 37.2% and 37.5% respectively (FIGURE 2.10). A bicarbonate-buffered M199 combination without phenol red was the medium of choice for subsequent experiments.

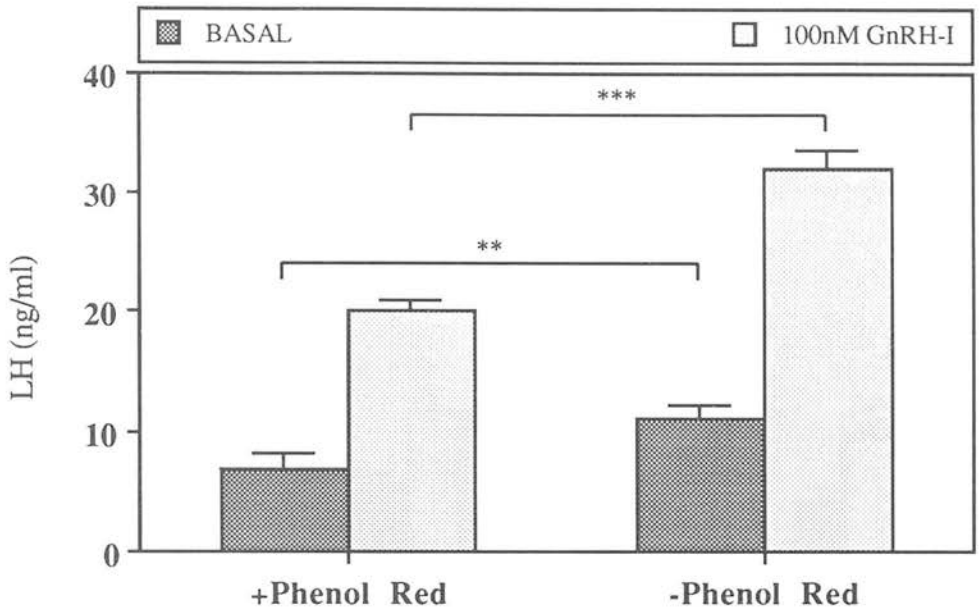


FIGURE 2.10: Effect of phenol red on the LH response of cultured pituitary cells to GnRH-I.
Pituitary cells (500,000/500 µl/well) from juvenile chickens were cultured in bicarbonate-buffered M199 ± phenol red (22.9 µM) for 48-h, rinsed in fresh medium and then incubated ± GnRH-I for 60-min. The medium was assayed for LH and the results presented as the mean ± sem; n = 8. **P<0.01, ***P<0.001.

2.6.4.3 Effects of culture period on cell appearance and function

The appearance of the collagenase-dispersed cells changed over a period of days in culture. After 24 - 72-hours, the ovoid - round cells became more flattened in appearance and formed cytoplasmic projections to neighbouring cells. These cell-to-cell associations are thought to be important in restoring the 'physiological' environment necessary for optimum pituitary cell function (Baes *et al.*, 1987; Denef *et al.*, 1989). Although the effect of cell density of the cultures on gonadotroph function was not studied, it is possible that a higher plating density would promote a more rapid formation of intercellular contacts thereby improving the functional properties of the cells.

The cell viability remained in excess of 90% for up to 72-hours and the cells retained their responsiveness to GnRH-I throughout this period (P<0.05 compared with the time-matched basal LH; FIGURE 2.11). Thus, GnRH-I stimulated a maximal fold-increase above basal of 4.5, 1.7, 2.9 and 3.0 in cells cultured for 5, 24, 48 and 72-hours respectively. However, the 48-hour cultures were more responsive to a lower concentration of GnRH-I, producing an increase of 2.3-times more LH release above basal, compared with 1.6, 1.7 and 1.8 for respectively 5, 24 and 72-hour cultures. Unless otherwise indicated, pituitary cells were cultured for 48-hours in the LH secretion studies.

Trypsin-dispersed cells cultured for between 6 - 72-hours also released LH in response to GnRH-I (FIGURE 2.12), however the basal and GnRH-I-stimulated LH responses decreased with time in culture (P<0.001 for all doses compared with the 6-hour cultures).

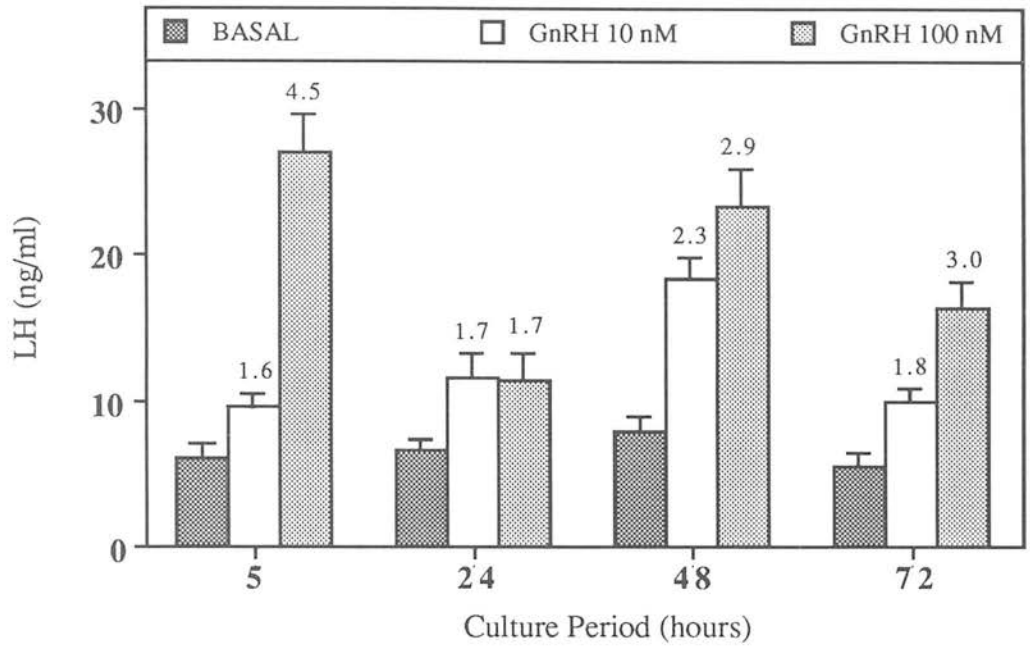


FIGURE 2.11: Effect of culture period on the LH response of pituitary cells to GnRH-I.
Pituitary cells (500,000/500 µl/well) from juvenile chickens were cultured for 5, 24, 48 and 72-h, rinsed in fresh medium and then incubated ± GnRH-I (10 or 100 nM) for 60-min. Results are presented as mean ± sem, n = 8. The fold-increase over the respective basal LH for each time-point is given above the GnRH-I-stimulated LH bars.

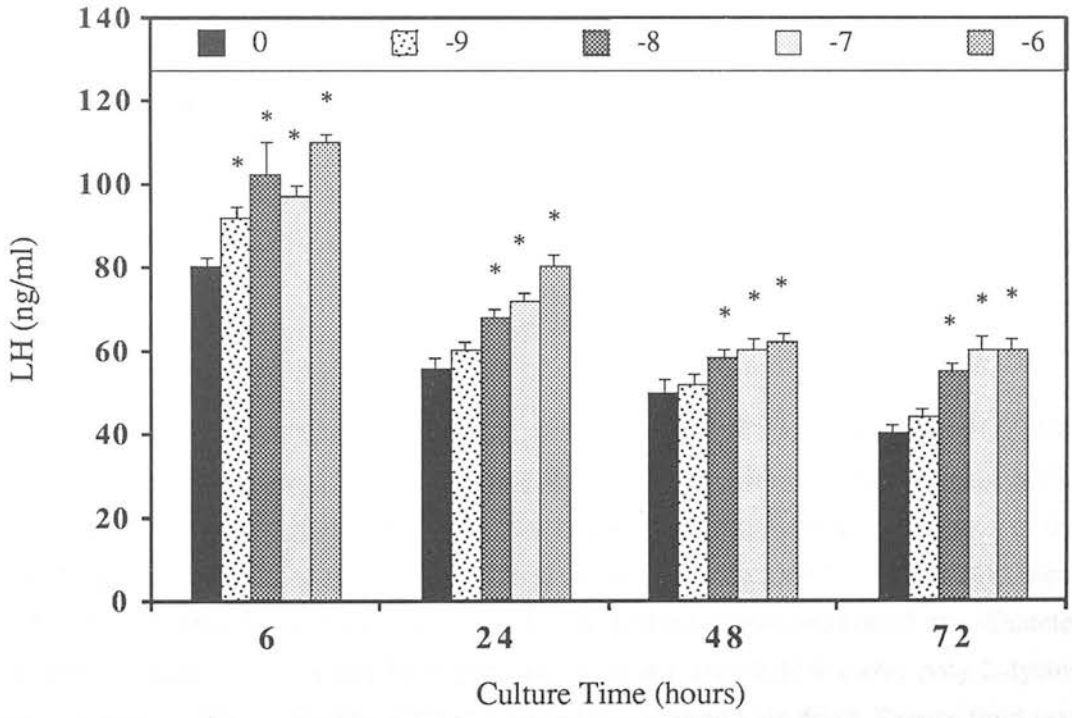


FIGURE 2.12: Effect of culture time on the dose-response relationship of GnRH-I in trypsin-dispersed cells.
The GnRH-I-stimulated (Log_{10} M) LH responses of trypsin-dispersed pituitary cells (500,000 cells/500 µl well) from juvenile chickens were measured after different periods in culture. Results shown as mean ± sem; *P<0.05 compared with time-matched basal LH, n = 8.

2.6.4.4 GnRH-I-stimulated LH response of steroid-treated pituitary cell cultures

The effects of the gonadal steroids, progesterone, testosterone, 17α-oestradiol and 17ß-oestradiol were investigated in cultured pituitary cells from juvenile chickens (with the exception of 17α-oestradiol, all steroids were of tissue culture grade; SIGMA). Stock solutions of 10 mM steroids were prepared in ethanol and diluted in M199-FCS; less than 0.1% (v/v) final ethanol concentration. After 3 - 4-hours in culture, the spent medium was removed and the plated cells gently rinsed in M199-FCS, and replaced with fresh medium containing the appropriate steroid. The cells were then returned to culture for a further 48-hours.

2.6.4.4.1 Cell stimulation protocol

Waste material and non-attached cells were removed from the wells, and the cells equilibrated with 200 or 500 µl fresh M199-NBCS or Buffer A-NBCS for 15-minutes at 37 or 41°C in an humidified atmosphere of 5% CO_2 and 95% air. The cells were rinsed in a further volume of medium which was then replaced by fresh medium in the presence or absence of drug and the cells returned to the incubator for 60-minutes. In stimulation studies with steroid-pretreated pituitary cells, the appropriate concentration of steroid was also included in the medium. Stock solutions of 1 mM GnRH-I were prepared in water and stored at -20°C. The activator of protein kinase C, 12-0-tetradecanoyl-13-phorbol acetate (TPA) was purchased from SIGMA and dissolved in DMSO (SIGMA).

In experiments to examine the effect of gonadal steroids on the cellular content of LH, the cells were rinsed with fresh medium and then lysed with 200 (48-well plates) or 500 µl (24-well plates) 1% Triton X-100 (v/v in water; SIGMA). This concentration of Triton X-100 did not interfere with the LH radioimmunoassay.

2.6.5 Immunocytochemical staining of gonadotrophs

Freshly dispersed anterior pituitary cells were prepared in M199-NBCS as described in SECTION 2.6.3.1 and blood cells removed by resuspending the cell pellet with 1 ml RBC-lysing buffer (SIGMA). After 1.5-minutes, 10 ml M199-NBCS was added and following centrifugation of the cells at 200 x **g** for 5-minutes at room temperature, the medium discarded. Pituitary cells were gently applied onto Thermanox plastic coverslips (LUX, Miles Laboratories) or 13 mm diameter glass coverslips (Merck) which had been previously coated with 0.01% (w/v) poly-L-lysine (molecular weight 70,000 - 150,000; SIGMA), rinsed in water and air-dried. Excess fluid was removed after 30-minutes and the coverslips rinsed gently in phosphate-buffered saline (PBS pH 7.4; see below). The cells were fixed with fresh 4% (w/v) paraformaldehyde (BIO-RAD) in PBS for 60-minutes. All incubations were at room temperature unless otherwise indicated. The coverslips were washed in PBS 3 x 15-minutes and incubated in 0.04% (v/v) hydrogen peroxide (SIGMA) prepared in PBS for 60-minutes. Coverslips were washed in PBS 3 x 15-minutes and

the cells permeabilised with 0.1% (w/v) saponin (Merck) in PBS for 10-minutes. Coverslips were washed in PBS 3 x 15-minutes and incubated with 5% normal goat serum (v/v; NGS, Scottish Antibody Production Unit) in PBS for 30-minutes to block non-specific binding of the second antiserum. The excess fluid was discarded and the residual removed by tissue paper. The coverslips were incubated overnight at 4°C in rabbit anti-chicken LH [3/3] (between 1-in-2500 and 1-in-20,000 dilution) in 5% NGS-PBS.

Phosphate-buffered saline (4 litres)

7.2 g $NaH_2PO_4.2H_2O$	61.15 g $Na_2HPO_4.2H_2O$
20 g NaCl	0.8 g $MgCl_2.6H_2O$

The pH was adjusted to 7.4.

Following 3 x 15-minute washes in PBS, the cells were incubated in 1-in-150 goat, anti-rabbit IgG-biotin conjugate (SIGMA) in PBS for 60-minutes. The coverslips were washed in PBS 3 x 15-minutes and incubated with streptavidin-biotinylated-horseradish peroxidase complex at 1-in-300 (Amersham International plc) in PBS for 60-minutes. Coverslips were then washed in PBS 3 x 15-minutes and then in 50 mM TRIS buffer (pH 7.4) for 5-minutes. A solution of 5 mg 3,3'-diaminobenzidine (SIGMA) in 9 ml TRIS buffer was prepared, to which 1 ml of 0.04% (v/v) hydrogen peroxide in TRIS was added. The solution was passed through a 0.2 µm filter (SIGMA) and incubated with the cells. The brown colour reaction was monitored using an inverted microscope.

To facilitate visualisation of the cells for counting purposes, the nuclei were stained with 0.5 µg/ml 4',6-diamidino-2-phenyl-indole (DAPI dihydrochloride, SIGMA) in MacIlvaines buffer (38.5 ml of 0.2 M Na_2HPO_4 and 61.5 ml of 0.1 M citric acid, pH 4.0) for 2-hours in the dark at room temperature. Excess fluid was drawn off with tissue paper and the glass coverslips were inverted and mounted in 1,4-diazo-bicyclo-2,2,2-octane (DABCO; SIGMA) in 90% (v/v) glycerol/PBS; plastic coverslips were mounted face-up in DABCO. The cells were examined under a microscope (OLYMPUS) equipped for fluorescence.

2.6.6 Membrane fluidity

The conditions for the technique were optimised for pituitary cells from sexually immature chickens. Pituitary glands were dispersed into cells by trypsin (SECTION 2.6.3.1). The cell density, the concentration of 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Cambridge Bioscience) and the temperature were examined for their effect on anisotropy

measurements. Stock solutions of 100 mM TMA-DPH were prepared in dimethyl sulphoxide (SIGMA) and stored at -70°C in the dark.

The cells were resuspended in 3 ml HEPES buffer (NaCl 154, KCl 5.4, HEPES 6.0, $CaCl_2$ 1.5, glucose 11.0 mM), labelled with TMA-DPH and continuously mixed by magnetic stirrer in quartz cuvettes (1 cm light path). The 'L'-format spectrofluorimeter (LS-50; Perkin Elmer) was used in *polarimetry* mode and anisotropy measurements were made at different temperatures. The temperature was controlled by circulating water through a four-position cuvette holder and was electronically monitored in a parallel cuvette containing 3 ml buffer, by a digital micro-thermistor. The excitation and emission wavelengths were 360 and 430 nm respectively with 10 nm slit widths.

The effect of gonadal steroids on membrane fluidity was investigated using pituitary cells from sexually immature broiler chickens (mixed sexes) cultured for 48-hours in the presence and absence of testosterone, progesterone, 17α-oestradiol or 17ß-oestradiol. Pituitary cells (5 million cells/4 ml M199-FCS) were dispensed into 35 mm petri dishes for 3 - 4-hours, before replacing with the appropriate steroid in fresh M199-FCS.

2.6.6.1 Optimisation of the conditions for anisotropy measurements

The cumulative effect of TMA-DPH concentration on the fluorescence intensity of pituitary cells from juvenile chickens is shown in FIGURE 2.13. An increase in fluorescence was measured on addition of 1 µM TMA-DPH (final concentration) to the cell suspension (already containing 0.01 µM TMA-DPH). One hundred micromolar TMA-DPH produced an immediate decrease followed by a slow rise in fluorescence. This was due to the direct addition of 20 µl of the dimethyl sulphoxide vehicle to the 2 ml cell suspension (FIGURE 2.13). A final concentration of 1 µM TMA-DPH was used in all subsequent experiments. Increments of temperature between 10 - 51°C decreased the fluorescence anisotropy of TMA-DPH-loaded pituitary cells, to which a straight line could be fitted (FIGURE 2.14).

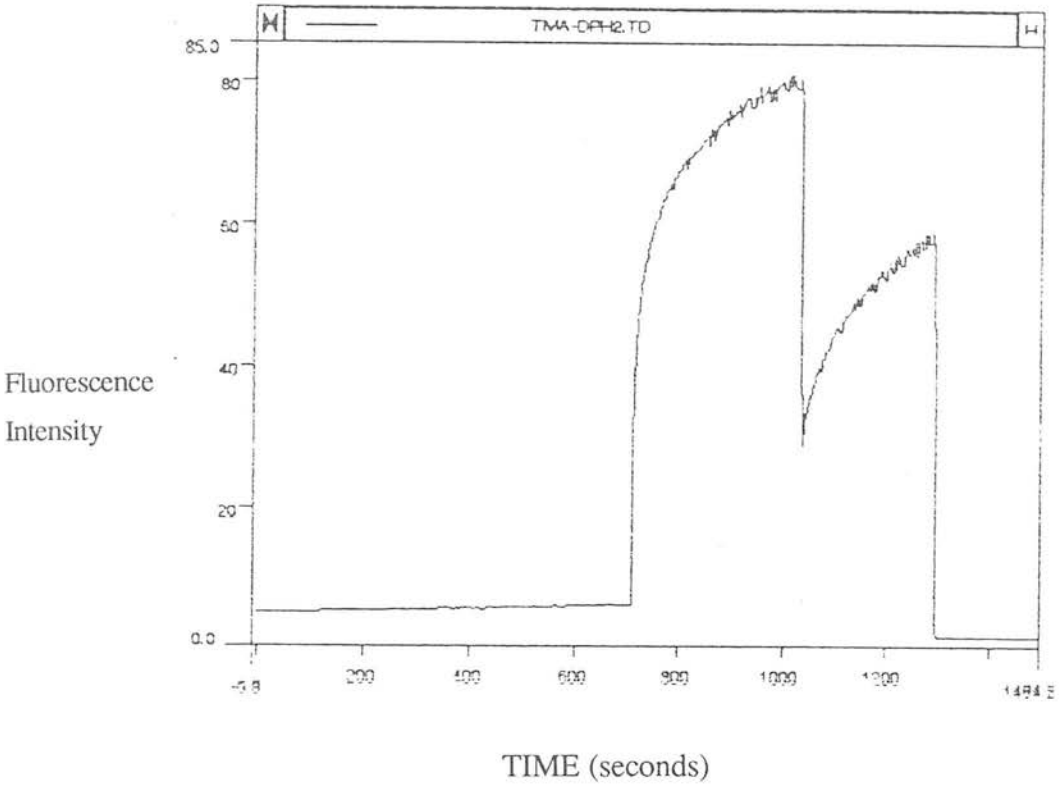


FIGURE 2.13: Cumulative effect of TMA-DPH concentration on the fluorescence intensity of pituitary cells.
The effect of increasing concentrations of TMA-DPH on the fluorescence intensity (arbitrary units) of freshly dispersed pituitary cells from juvenile chickens (mixed sexes; 1 million cells/ml) was measured at 37°C.

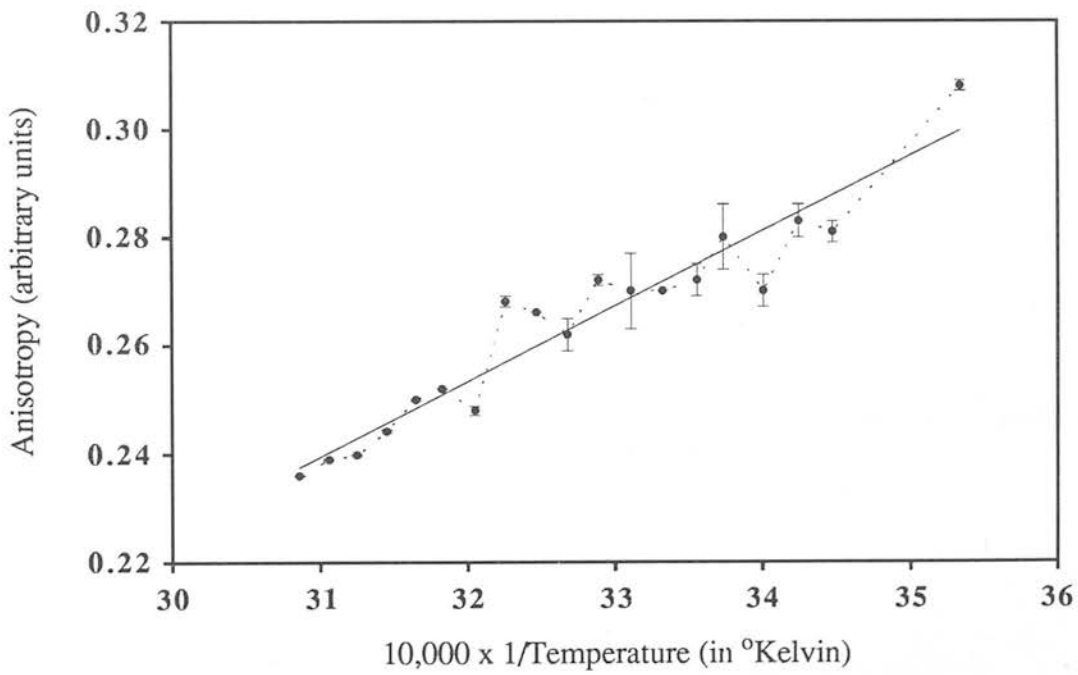


FIGURE 2.14: Relationship between anisotropy and temperature.
Pituitary glands from juvenile chickens (mixed sexes) were dispersed and immediately loaded with 1 μM TMA-DPH for anisotropy measurements (arbitrary units; n = 4) between 10 and 51°C (expressed as 10,000 x 1/absolute temperature). Straight line fitted by regression (correlation coefficient = 0.956).

Addition of TMA-DPH to pituitary cells rapidly increased the fluorescence intensity to 90% of maximum within 3-minutes, which was virtually stable for at least 20-minutes (FIGURE 2.15). Consequently, all subsequent measurements were made between 10 and 20-minutes after addition of the probe. Fluorescence intensity increased linearly with increasing density of TMA-DPH-loaded cells between 250,000 - 1,000,000 cells/ml (FIGURE 2.16); an intermediate density of 500,000 cells/ml was selected for later studies.

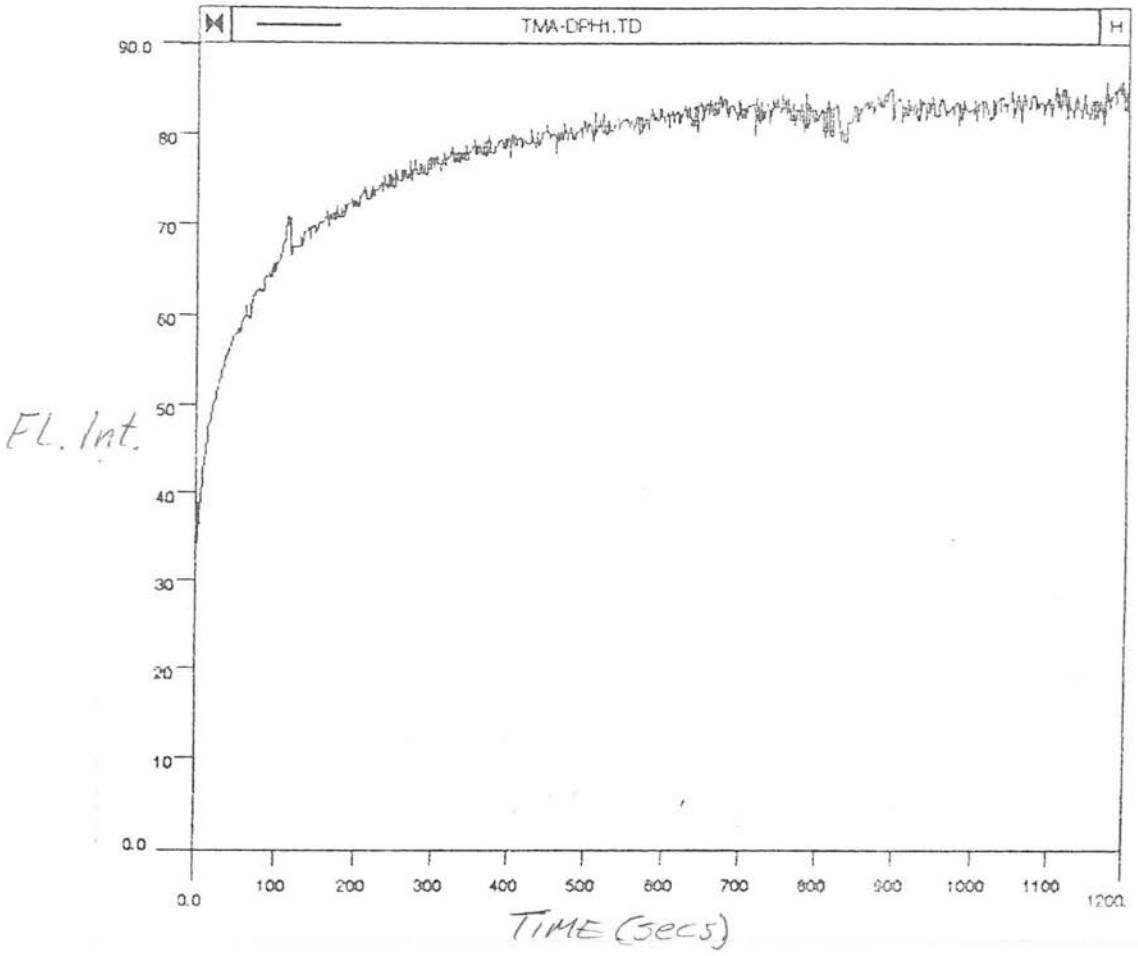


FIGURE 2.15: Time-course of TMA-DPH incorporation into pituitary cells.
Pituitary glands from juvenile chickens were dispersed and the cells immediately loaded with 1 μM TMA-DPH for fluorescence intensity measurements (arbitrary units) at 37°C.

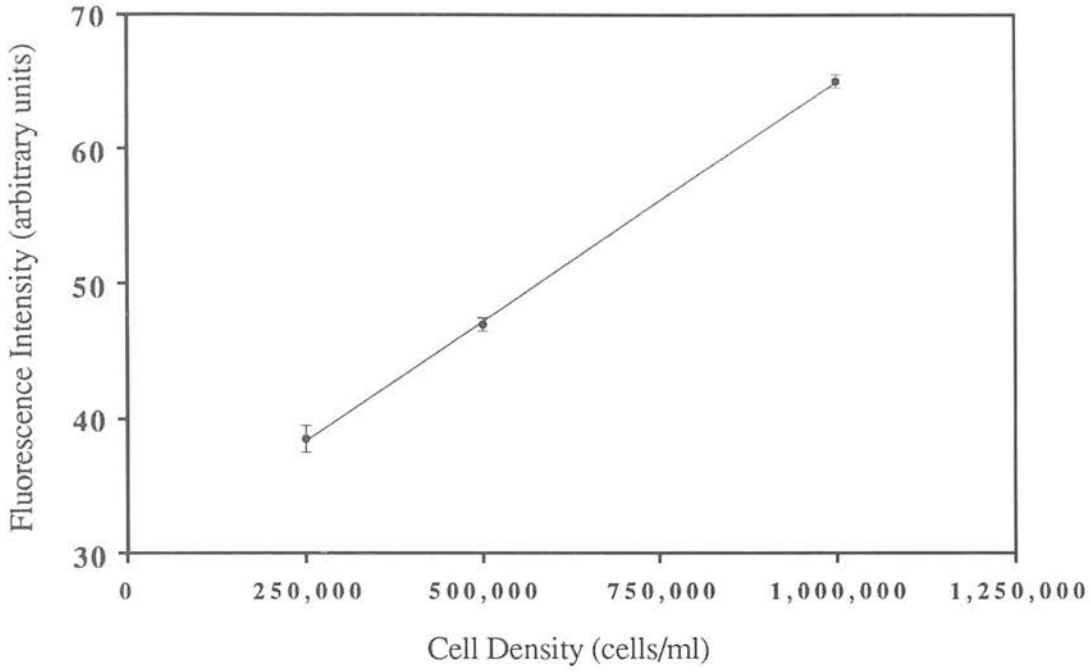


FIGURE 2.16: Effect of cell density on fluorescence intensity.
Pituitary glands from juvenile chickens were dispersed and the effect of TMA-DPH-loaded cell density on the fluorescence intensity (arbitrary units; n = 4) measured at 37°C.

2.7 DATA PRESENTATION AND ANALYSIS

All data were processed by Apple Macintosh® (Apple Computer Inc) and unless otherwise indicated, were expressed as the mean ± standard error of the mean (sem). Determination of statistical significance of data obtained from radioimmunoassays was by Student's unpaired *t*-test (StatWorks®; Cricket Software) or analysis of variance (StatView-II™, SuperAnova™) of the $\log_{10}$-transformed data, where $P<0.05$ was considered significant. Where appropriate, analysis of data was by simple linear regression (StatWorks®) and measurements of area-under-the-curve (AUC) by MUNRO® (Zaristow Software).

3.1 INTRODUCTION

Gonadotrophin-releasing hormone-I (GnRH-I) stimulates a sexually differentiated LH response in the adult chicken (Sharp *et al.*, 1987). Thus relative to the cockerel, the laying hen is less sensitive and less responsive to GnRH-I, and the duration of the elevated concentration of LH is longer (Sharp *et al.*, 1987). Furthermore, the resting concentration of plasma LH in the laying hen is lower than in the adult cockerel (Sterling & Sharp, 1984; Sharp *et al.*, 1987). A sex difference is also present, though less pronounced, in the juvenile chicken; i.e. a greater magnitude of LH release in the male compared with the female, but a similar sensitivity and time-course of LH release in response to GnRH-I (Wilson *et al.*, 1989). In this chapter, the sex difference in the LH response of the adult domestic chicken to GnRH-I is further characterised and related to concentrations of hypothalamic GnRH-I and GnRH-II, pituitary LH and plasma steroid hormones, in order to identify the possible mechanisms involved in this sex difference.

3.2 RESULTS

3.2.1 Hormones of the hypothalamic-pituitary-gonadal axis

3.2.1.1 Sexual differences in hypothalamic contents of GnRH-I and GnRH-II in the adult and juvenile chicken

A comparison was made between the amounts of GnRH-I and GnRH-II in the mediobasal (MBH) and preoptic area (POA) of the hypothalamus from adult and juvenile cockerels and hens.

The adult cockerel and hen had similar amounts of GnRH-I and GnRH-II in the MBH and POA (TABLE 3.1). However the MBH and POA tissue from adult cockerels weighed more ($P<0.05$) than those from laying hens of the same age (TABLE 3.2). The concentrations of GnRH-I and GnRH-II in the MBH and POA of adult chickens were therefore sexually differentiated when corrected for the sex differences in tissue weight (FIGURE 3.1). There were no differences in weights of MBH or POA between sexually immature males and females. The amount of GnRH-II in the POA of the juvenile male was significantly different ($P<0.05$) from that of the juvenile female, but not when the data were expressed as pg GnRH-II/mg tissue weight (FIGURE 3.1).

TABLE 3.1: Comparison of the contents of GnRH-I and GnRH-II in the MBH and POA of adult and juvenile chickens.

		GnRH-I (ng/tissue)		GnRH-II (pg/tissue)	
		MBH	POA	MBH	POA
ADULT	Male	13.4 ± 1.5	3.1 ± 0.3	325.8 ± 32.1	1003.9 ± 92.0
	Female	16.5 ± 1.6	3.7 ± 0.4	387.7 ± 41.8	1200.0 ± 81.5
JUVENILE	Male	2.8 ± 0.3	1.0 ± 0.1	199.4 ± 26.7	349.0 ± 27.0
	Female	2.7 ± 0.3	1.0 ± 0.1	276.4 ± 34.9	445.6 ± 18.2**

Adult and juvenile chickens were 21 and 7.5-weeks old respectively. **P<0.01 compared with the respective tissue of the age-matched male (n = 8 per sex). Note difference in units.

TABLE 3.2: Comparison of body weights and the weights of the MBH and POA of the adult and juvenile chicken

		MBH weight (mg)	POA weight (mg)	Body weight (kg)
ADULT	Male	55.3 ± 4.5	75.4 ± 2.8	2.8 ± 0.07
	Female	*40.0 ± 3.0	*66.4 ± 3.1	***1.8 ± 0.04
JUVENILE	Male	40.4 ± 2.7	43.9 ± 6.2	0.8 ± 0.02
	Female	NS47.3 ± 4.0	NS49.7 ± 6.8	**0.7 ± 0.02

Adult and juvenile chickens were 21 and 7.5-weeks old respectively. NS = not significantly different, *P<0.05, **P<0.01, ***P<0.001 compared with age-matched male (within column; n = 8 per sex)

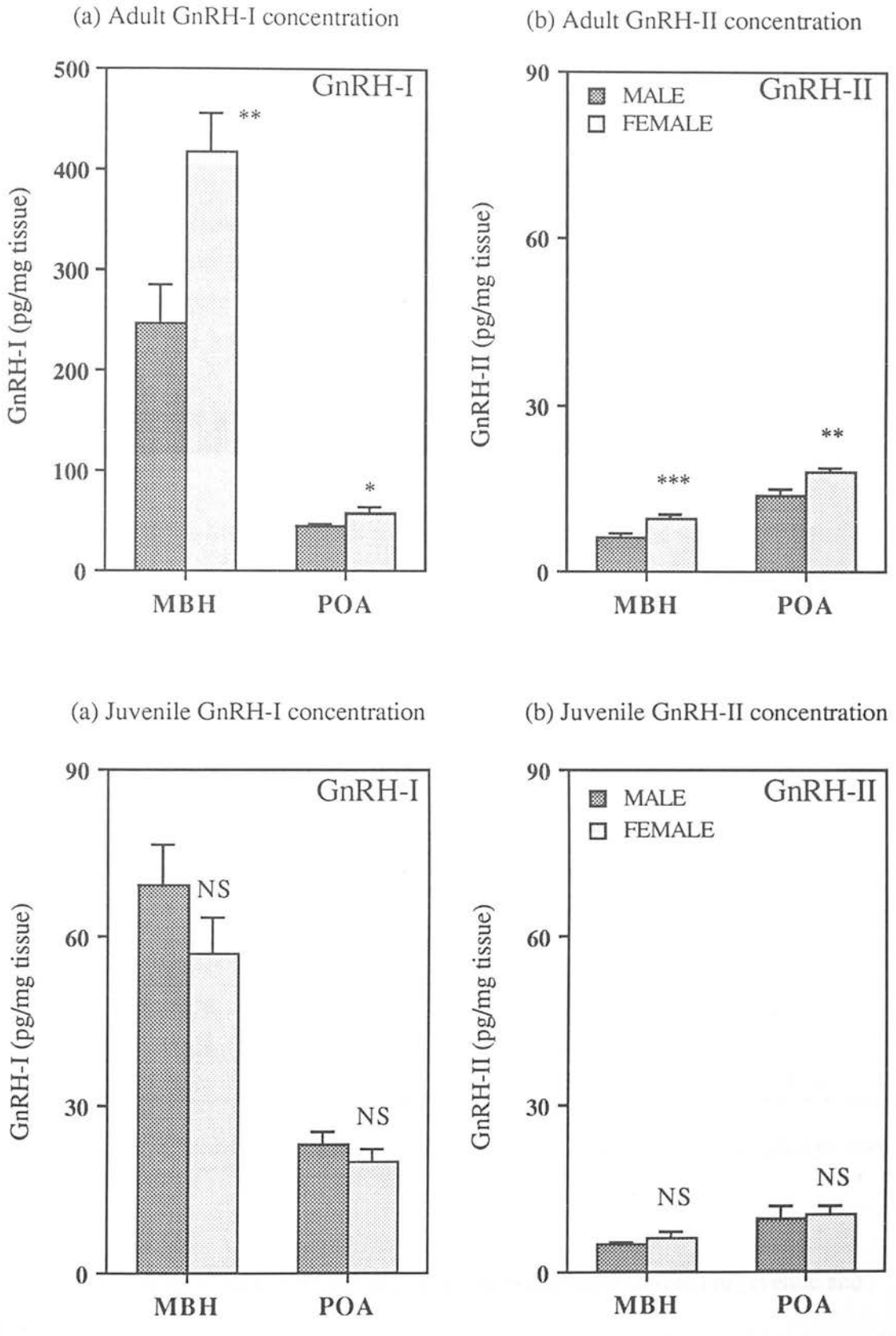


FIGURE 3.1: Comparison of the concentrations of GnRH-I and GnRH-II in the preoptic area (POA) and mediobasal hypothalamus (MBH) of adult and juvenile chickens.

POA and MBH brain areas were collected from 21 and 7.5-week old chickens (n = 8 per age per sex) for (a) GnRH-I and (b) GnRH-II assays. *P<0.05, **P<0.01, ***P<0.001 compared with the respective brain regions of the age-matched male. Note the different scale on the y-axis of adult GnRH-I graph.

The concentration of GnRH-I was greater in the MBH than in the POA ($P<0.001$; FIGURE 3.1), and this difference was greater in adult than in juvenile cockerels and hens. Thus on reaching sexual maturity, the concentrations of GnRH-I in the MBH rose by 3.6 and 7.2-fold in cockerels and hens respectively, whereas the POA concentrations of GnRH-I rose by 1.8 and 2.6-fold. In the cockerel, on reaching adulthood, there were no significant differences before and after puberty, between the concentrations of GnRH-II (pg/mg tissue) in the MBH (juvenile = 4.9 ± 0.5, adult = 6.0 ± 0.7) or the POA (juvenile = 9.6 ± 2.1, adult = 13.8 ± 0.9). The concentration of GnRH-II (pg/mg tissue) was higher than in laying hens (21-weeks-old) than in juvenile hens (7.5-weeks-old) in the MBH (juvenile = 6.2 ± 0.9, adult = 9.7 ± 0.7; $P<0.05$) and in the POA (juvenile = 10.2 ± 1.2, adult = 18.1 ± 0.7; $P<0.001$).

3.2.1.2 Sex difference in content of LH in anterior pituitary glands from adult (21-week-old) chickens

Anterior pituitary glands from laying hens contained ten-times less LH and weighed 37% less than pituitary glands from adult cockerels (TABLE 3.3). There was no sex difference between the weights of pituitary glands from juvenile chickens. The concentrations of pituitary LH in pituitary glands from adult cockerels and juvenile males and females were not significantly different from each other.

TABLE 3.3: Comparison of the LH contents of anterior pituitary glands from adult and juvenile chickens

		Pituitary LH Content (µg/pituitary)	Pituitary Weight (mg)	Pituitary LH Concentration (µg/mg)
ADULT	**Male**	14.3 ± 3.2	14.7 ± 0.8	0.96 ± 0.20
	Female	$1.5 \pm 0.1^{***}$	$9.3 \pm 0.3^{***}$	$0.16 \pm 0.01^{***}$
JUVENILE	**Male**	6.1 ± 0.8	6.4 ± 0.4	0.86 ± 0.09
	Female	5.6 ± 0.7^{NS}	5.9 ± 0.2^{NS}	1.10 ± 0.16^{NS}

Pituitary glands were collected from 21 and 7.5-week old cockerels and hens (n = 8 per sex per age). NS = not significantly different, ***P<0.001 compared with age-matched male.

3.2.1.3 Sex differences in the concentrations of plasma gonadal steroids in juvenile and adult chickens

The concentration of 17ß-oestradiol was sexually differentiated in both adult and juvenile chickens (TABLE 3.4). There was no significant difference between the progesterone concentrations of the juvenile male and female, and progesterone was undetectable in the adult cockerel.

TABLE 3.4: Sex and age-related differences in the baseline concentrations of plasma progesterone and 17ß-oestradiol in juvenile and adult chickens.

		17ß-oestradiol (pg/ml)	Progesterone (pg/ml)
ADULT	**Male**	$90.5 \pm 25.8^{\dagger\dagger}$	$<39^{\dagger}$
	Female	$234.0 \pm 21.5^{*}$	$225.5 \pm 90.3^{*}$
JUVENILE	**Male**	$67.8 \pm 4.8^{***}$	106.9 ± 12.3^{NS}
	Female	154.4 ± 12.6	83.6 ± 9.9

Blood samples collected at 5 - 6 -h before lights off. Minimum detectable concentration of progesterone was $39^{\dagger}$ pg/ml. NS = not significantly different, *P<0.05, ***P<0.001 compared with juvenile female (n = 5 - 13). ††P<0.01 compared with age-matched female and not significantly different from juvenile male.

3.2.2 Sex differences in the plasma LH response of adult chickens to GnRH-I

3.2.2.1 Preliminary studies on the pituitary responsiveness to GnRH-I in adult cockerels and hens

A preliminary study compared the magnitude of LH release in adult cockerels and hens to establish the doses of GnRH-I for later studies. Doses of 0.1 and 0.5 µg GnRH-I/kg, and 5 and 20 µg GnRH-I/kg were selected for the adult cockerel and the hen respectively, based on previous observations (Sterling & Sharp, 1984; Sharp *et al.*, 1987; Wilson *et al.*, 1989). A supramaximal dose of 20 µg GnRH-I/kg was also administered to adult cockerels.

The baseline concentration of plasma LH in laying hens was lower than in cockerels of the same age (P<0.001, n = 18; TABLE 3.5). Compared with the adult cockerel, 10-times more GnRH-I was required to elevate significantly (P<0.05, n = 6) the concentration of plasma LH in the laying hen. The incremental change in plasma LH (ΔLH) 10-minutes after injection of 20 µg GnRH-I/kg in laying hens was less than in cockerels (P<0.001; TABLE 3.5). There was no difference between the ΔLH released in response to 0.5 µg GnRH-I/kg and 20 µg GnRH-I/kg in adult cockerels.

TABLE 3.5: Comparison of the baseline and incremental changes in concentrations of plasma LH in adult (14-month old) cockerels and hens before and after injection of GnRH-I.

	Baseline LH (ng/ml)	ΔLH (ng/ml) 10-min after GnRH-I Dose of GnRH-I (μg/kg)			
		0.1	0.5	5	20
MALE	6.3 ± 0.3	3.7 ± 1.0	18.8 ± 2.5	NT	18.3 ± 2.5
FEMALE	3.0 ± 0.1***	NT	NT	1.3 ± 0.5	1.8 ± 0.5***

ΔLH at 10-mins after GnRH-I injection = LH_{GnRH-I} - LH_{saline}. NT = not tested. ***P<0.001 compared with male (n = 6 per sex).

3.2.2.2 Sex differences in the pituitary LH response of adult and juvenile cockerels and hens to a supramaximal dose of GnRH-I

More GnRH-I is required to stimulate LH secretion in the laying hen than in the cockerel because of the difference in sensitivity to GnRH-I between the adult sexes (Sharp *et al.*, 1987). The adult male and female chicken have similar plasma half-lives of GnRH-I (Sharp *et al.*, 1987), which means that the larger dose of GnRH-I given to laying hens will circulate for longer than the dose given to adult cockerels. The possibility that this explains the prolonged release of LH in laying hens was explored by comparing the effect on LH release of a female-maximally stimulatory dose of GnRH-I (20 μg/kg) in adult cockerels and laying hens.

The concentration of plasma LH was increased (P<0.05) within 2-minutes of GnRH-I injection in adult cockerels and laying hens (FIGURE 3.2). The concentration of plasma LH returned to baseline values in the male by 60-minutes after GnRH-I injection. The LH response of the female had not returned to baseline values by 60-minutes but did so after 120-minutes (not significantly different from control). In adult cockerels, the rate of decline from maximum concentrations to half ΔLH following 20 μg GnRH-I/kg was 23.6 ± 3.8 minutes (measured by linear regression analysis of LH concentration versus Log_{10} time after injection; correlation coefficient ranged between 0.963 - 0.995). Corresponding times for the laying hen ranged between 22-minutes to 1.7 x 10^{10}-minutes (correlation coefficient = 0.008 - 0.419; a mean time of 47-minutes was calculated from the mean decline in concentration of LH).

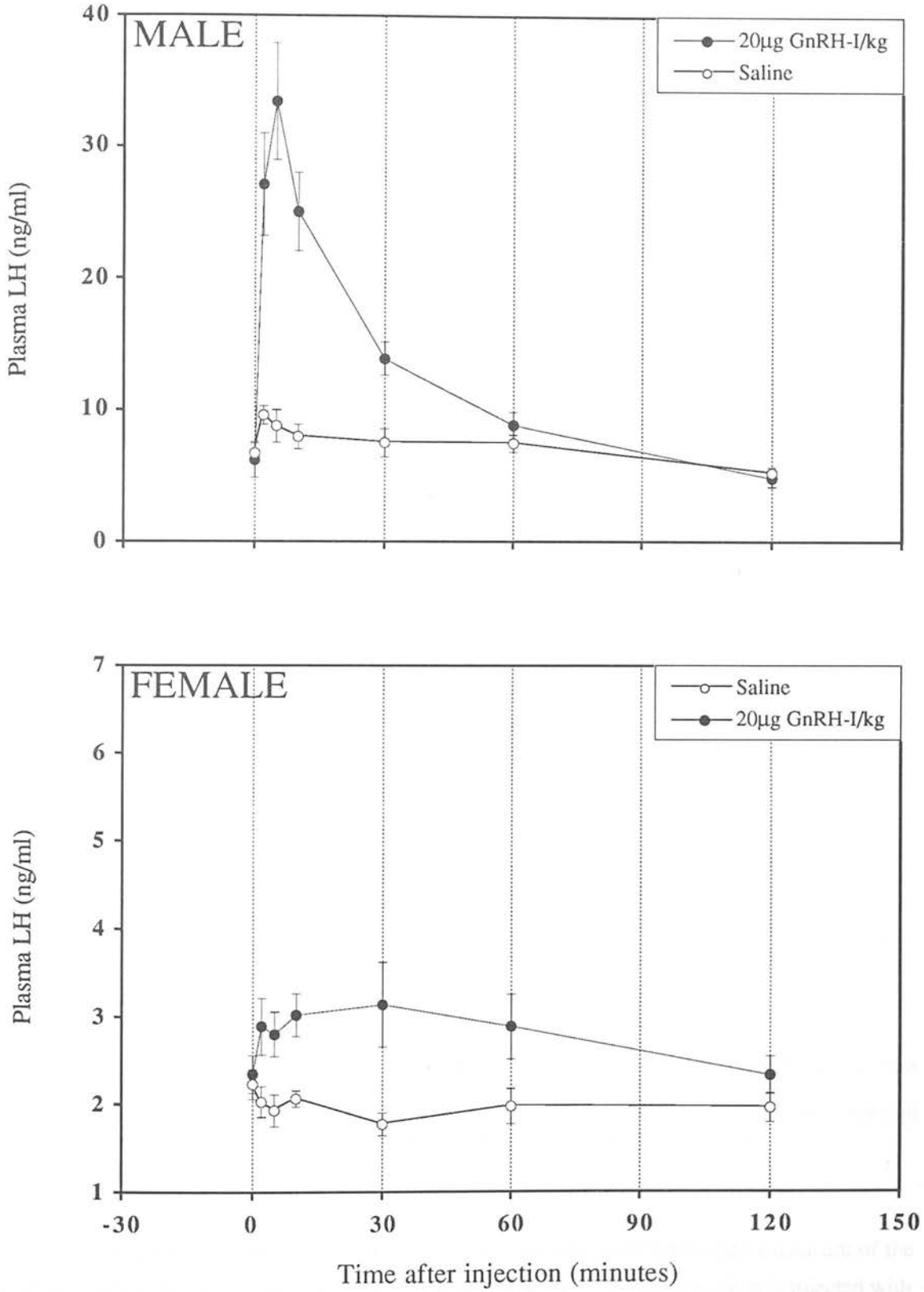


FIGURE 3.2: Effect of an intravenous injection of GnRH-I on the concentration of plasma LH in adult cockerels and hens.
Adult cockerels and laying hens (n = 8) were injected intravenously with saline or 20 µg of GnRH-I/kg and blood samples collected over 120-min. NS = not significantly different. Note the different scales on the y-axes.

A maximally stimulatory dose of 0.5 μg GnRH-I/kg in juvenile chickens was selected from previous studies (Wilson *et al*, 1989; Nakamura *et al*, 1991). Qualitatively similar increases in the concentrations of plasma LH were seen in juvenile male and female chickens after injection of 0.5 μg GnRH-I/kg (FIGURE 3.3). The resting concentration of plasma LH in the juvenile male (4.6 ± 0.6 ng/ml) was significantly higher than in the juvenile female (3.4 ± 0.4 ng/ml; $P<0.05$, n = 15/16), and the peak concentration of plasma LH in the male (29.6 ± 2.6 ng/ml) was greater than that of the female (20.3 ± 2.5 ng/ml; $P<0.05$, n = 7/8). The stimulated LH responses of the sexually immature male and female returned to control levels by 60-minutes after GnRH-I injection. The rate of decline to half ΔLH concentrations was 7.9 ± 0.3-minutes in the male, and 9.0 ± 0.3-minutes in the female ($P<0.05$).

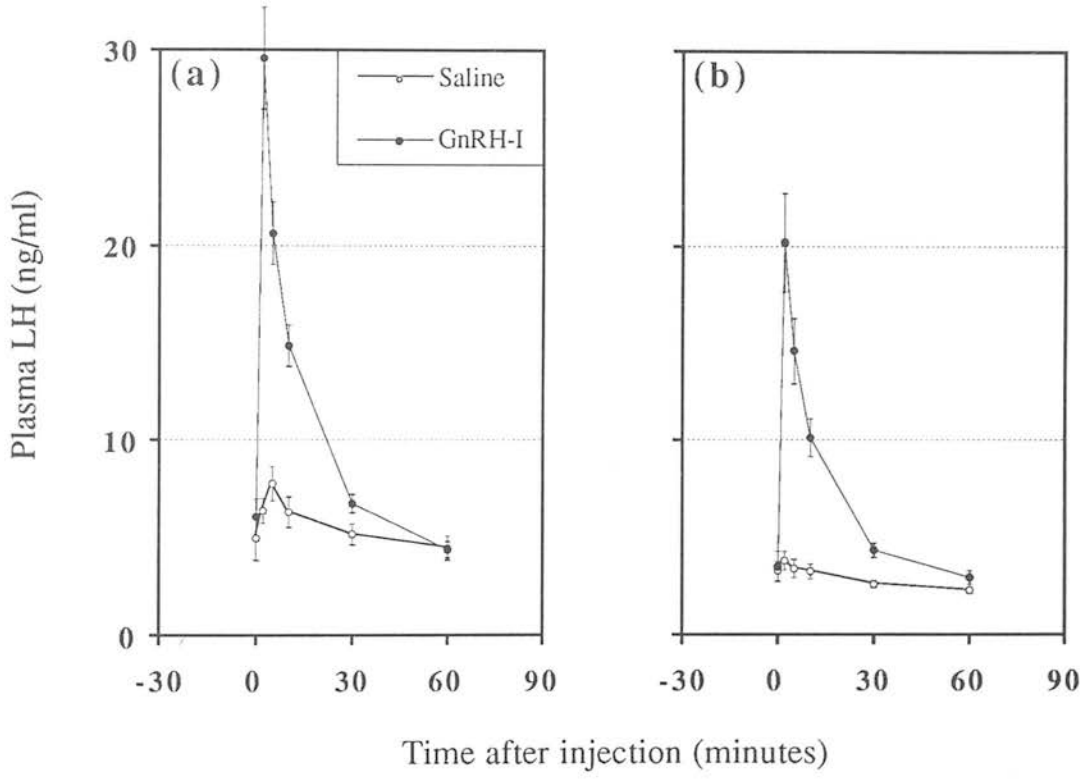


FIGURE 3.3: Effect of GnRH-I on the plasma LH response of juvenile male and female chickens.
Sexually immature 7.5-week old (a) male and (b) female chickens (n = 7/8 per sex) were injected intravenously with saline or 0.5 μg GnRH-I/kg and blood samples collected over 60-min.

Calculation of area-under-the-curve (AUC) of the plasma LH profile provides a measure of the amount of LH released over the experimental period. The AUCs for adult cockerels injected with saline or 20 μg GnRH-I/kg were significantly greater than the corresponding values for the laying hen (both $P<0.001$). The GnRH-I-stimulated ΔAUC (LH AUC_{GnRH-I} minus LH AUC_{saline}) was also greater in adult cockerels than in laying hens ($P<0.001$; TABLE 3.6). Despite the difference in the magnitude of peak LH concentrations after injection of GnRH-I, the total amount of LH released (ΔAUC) in the juvenile male and female was not significantly different (TABLE 3.6).

TABLE 3.6: Area-under-the-curve (AUC) of LH profiles from adult and juvenile chickens injected with saline or GnRH-I.

	Juvenile[A]		Adult[B]	
	Male	Female	Male	Female
Saline	393 ± 44	226 ± 19**	684 ± 102	122 ± 27***
GnRH-I	678 ± 54	512 ± 24*	1417 ± 135	335 ± 77***
∅AUC	285 ± 54	286 ± 24[NS]	733 ± 135	213 ± 77***

AUC of LH profiles after saline or GnRH-I injection calculated by MUNRO®. ΔAUC of LH = [AUC_{GnRH-I} minus AUC_{saline}]. [A]Juvenile (7.5-weeks old) and [B]adult (30-weeks old) chickens received respectively 0.5 and 20 µg GnRH-I/kg. AUCs of adults and juveniles are not comparable because AUCs were measured over different times using different GnRH-I doses; [A]60-min (n = 7/8), [B]120-min (n = 8). NS = not significantly different, *P<0.05, **P<0.01 and ***P<0.001 compared with age-matched males.

3.2.3 Plasma half-life of ^{125}I-LH in adult hens and cockerels

The sex difference in time-course of the LH response to GnRH-I in adult chickens may be due to differences in the degrading mechanisms for LH. This possibility was investigated by measuring the disappearance of ^{125}I-LH from plasma. There was no difference between the plasma half-life of ^{125}I-LH in the hen (16.7 ± 2.7-minutes) and the male (21.9 ± 3.1-minutes; FIGURE 3.4).

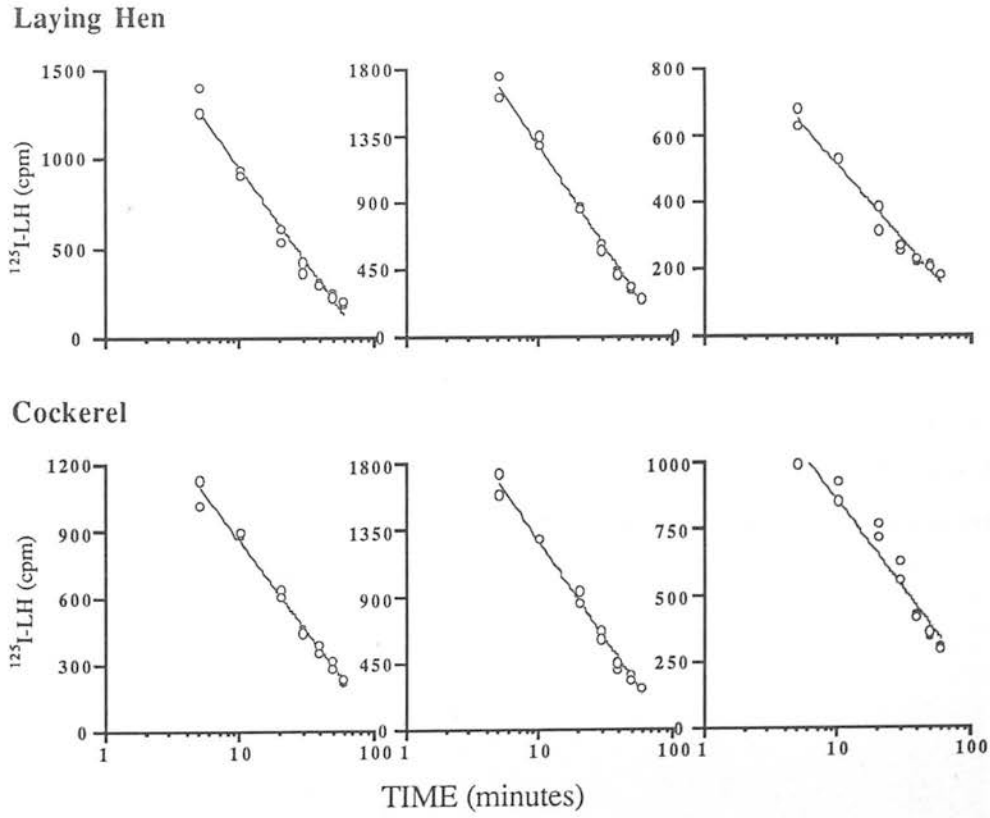


FIGURE 3.4: The disappearance of ^{125}I-LH from plasma in adult cockerels and hens. Chickens were injected intravenously with ^{125}I-LH (n = 3 per sex) and timed bleeds collected for 60-min. The amount of ^{125}I-LH in plasma (cpm) is plotted against time (on Log_{10} scale), and a straight line fitted by linear regression. Correlation coefficients ranged between 0.974 - 0.996.

3.2.4 Sex differences in baseline plasma LH concentration after passive immunisation with anti-GnRH-I serum

The sex difference in response to GnRH-I could reflect differences in LH release from the anterior pituitary gland which are not directly dependent on GnRH-I release. This possibility was investigated by measuring plasma LH after passive immunisation with anti-GnRH-I serum.

Immunoneutralisation of circulating GnRH-I reduced the concentration of plasma LH within 30-minutes of injection in cockerels ($P<0.05$, $n = 6$; FIGURE 3.5). The LH concentration returned to the pre-injection level by 24-hours after injection. Anti-GnRH-I serum did not depress the baseline concentration of plasma LH or egg-laying in adult hens. Non-immune sheep serum (NSS) had no significant effect on the LH concentrations of either sex.

The amount of anti-GnRH-I in plasma was also evaluated (FIGURE 3.5). The peak concentrations of anti-GnRH-I were calculated to bind a maximum of 1050 pg GnRH-I/ml of plasma in the cockerel and 1250 pg GnRH-I/ml of plasma in the hen.

3.2.5 Immunoneutralisation of GnRH-I in the laying hen during the preovulatory LH surge

The lack of effect of a single dose of anti-GnRH-I may be due to insufficient circulating levels and rapid clearance of antibody. Repeated injections of anti-GnRH-I were used during the preovulatory surge of LH. There was no effect on the concentration of LH 2-hours after the first injection of anti-GnRH-I (FIGURE 3.6). Multiple injections of anti-GnRH-I serum reduced the magnitude of the preovulatory LH surge ($P<0.001$, $n = 6$; ANOVA) but did not block egg-laying. Both peaks of LH occurred at 17:00-h. At the end of the experiment, the concentration of plasma LH was significantly lower in the anti-GnRH-I-injected hens compared with the controls ($P<0.05$, $n = 6$; *t*-test). The percentage-bound ^{125}I-GnRH-I increased in a stepwise manner coincident with the period after each injection. Plasma from NSS-injected birds contained less than 1% binding activity to GnRH-I throughout the experiment.

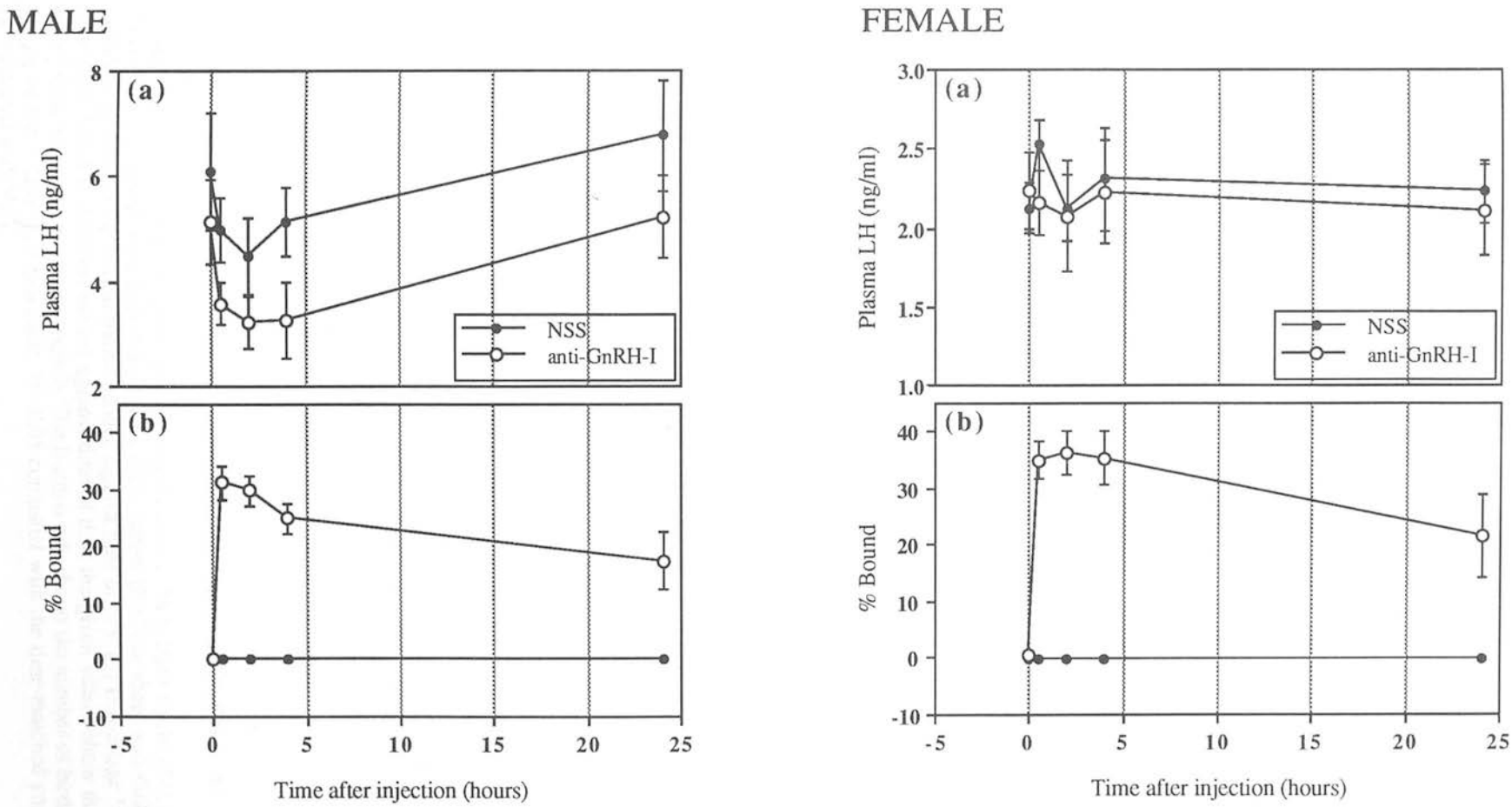


FIGURE 3.5: Effect of passive immunisation against GnRH-I on the concentration of plasma LH in adult cockerels and laying hens.

Adult chickens (n = 6 per sex) received an intravenous injection of 2 ml non-immune sheep serum (NSS) or sheep anti-chicken GnRH-I serum, and blood samples collected over 24-h. Measurements were made on (a) the LH concentration and (b) the levels of anti-GnRH-I in plasma (percent binding to ^{125}I-GnRH-I of 1-in-200-diluted plasma). Results are given as mean ± sem.

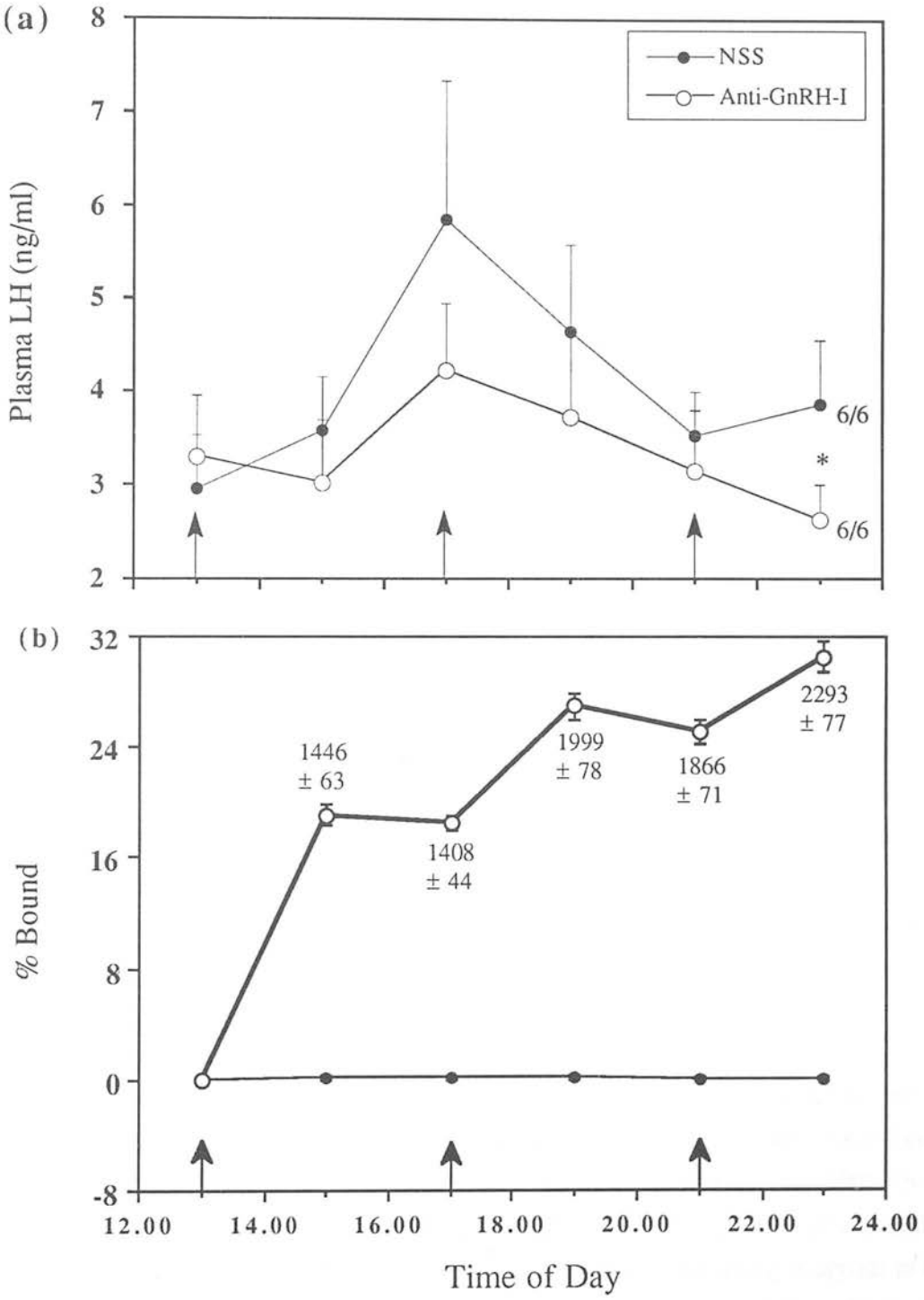


FIGURE 3.6: Effect of passive immunisation against GnRH-I on the preovulatory LH surge.

Laying hens (n = 6 per treatment group) entrained to a 28-h light cycle (14L:14D) were injected intravenously (arrows) with 2 ml non-immune sheep serum (NSS) or sheep anti-GnRH-I antibody (Anti-GnRH-I). (a) Plasma LH concentration plotted against time of day. (b) Percentage ^{125}I-GnRH-I bound to plasma (at 1-in-400 dilution) plotted against time of day; the given values show the theoretical mean ± sem of GnRH-I bound to plasma (pg/ml). The fraction n/6, shows the number of birds out of 6, which laid an egg on the 3 days after treatment. *P<0.05 compared with the time-matched concentration of LH of birds injected with NSS.

3.3 DISCUSSION

3.3.1 Sex differences in the concentrations of hormones in the hypothalamic-gonadotroph-gonadal axis

The sex differences in concentrations of hormones of the hypothalamic-gonadotroph axis were fully established after sexual maturation and therefore could be due to the sex differences in the concentrations of plasma 17ß-oestradiol and testosterone. An alternative possibility is that the sex difference in the hypothalamic-gonadotroph axis is independent of gonadal steroids. This possibility is suggested by the higher concentration of plasma LH in male quail than in female quail (Nicholls *et al.*, 1973; Davies, 1976) which persists even after gonadectomy (Urbanski & Follett, 1982). However these birds had undergone one reproductive cycle before gonadectomy, and the previous exposure to increased concentrations of gonadal steroids in plasma may have induced sexual differentiation of the hypothalamic-gonadotroph axis. This seems likely because no sex difference was seen in the photostimulated release of LH in quail gonadectomised as juveniles (Gibson *et al.*, 1975). It is probable therefore that the sexually differentiated concentrations of plasma LH in the adult chicken are determined by the differences in concentration of gonadal steroids between the two sexes.

3.3.1.1 Sex difference in the hypothalamic contents of GnRH-I and GnRH-II

Two important conclusions can be drawn from the measurements of concentrations of GnRH-I and GnRH-II in the MBH and POA. Firstly that there is a maturation-related increase in the concentrations of GnRH-I and GnRH-II in the hen but only of GnRH-I in the cockerel, and secondly that there is a sex difference in the concentrations of both peptides in adult (21-weeks of age) but not juvenile chickens (7.5-weeks of age).

There were increases in the concentrations of GnRH-II in the MBH and POA of the hen but not in the cockerel on reaching sexual maturity. The absence of a maturational change in the hypothalamic concentration of GnRH-II in cockerels agrees with a previous report by Sharp *et al.* (1990). These results suggest that GnRH-II may be physiologically important in the regulation of a sexually differentiated function associated with sexual maturation. However these changes appear to be independent of the concentration of gonadal steroids in plasma because castration of juvenile cockerels, or treatment with testosterone or 17ß-oestradiol result in changes in hypothalamic GnRH-I content but do not affect the content of GnRH-II in the posterior hypothalamus (Sharp *et al.*, 1990; Wilson *et al.*, 1990b). It is not certain from these observations what function GnRH-II might serve in the hen but Wilson *et al.* (1990a) suggest that GnRH-II may be involved in some event between the preovulatory release of LH and ovulation. Although GnRH-II probably acts as a neurotransmitter in the brain, its physiological importance in cockerels remains to be investigated. Maturational increases were found in the concentrations of GnRH-I in the hypothalamus of cockerels and hens which could be associated with the increasing levels of steroids in plasma (see

SECTION 3.3.1.3). The increase in the amount of GnRH-I in the MBH after the onset of sexual maturity confirms previous reports in the hen (Knight *et al.*, 1985) and cockerel (Knight, 1983; Stansfield & Cunningham, 1988). In the hen, there is also a concomitant but non-parallel increase in the K^+-releaseable GnRH from these tissues which is most pronounced after the onset of lay (Knight *et al.*, 1985).

The higher concentration of hypothalamic GnRH-I in hens than in cockerels on reaching sexual maturity may be required in laying hens to generate a preovulatory surge of LH. This requirement would be essential in order to overcome the lower sensitivity of the pituitary gland of laying hens to GnRH-I compared with the pituitary gland of adult cockerels (see SECTION 3.3.3), and also accounts for the decrease in hypothalamic GnRH-I content during the preovulatory surge of LH (Knight *et al.*, 1984). Since the release of GnRH-I is suppressed by 17ß-oestradiol and leads to an accumulation of GnRH-I in the posterior hypothalamus (Wilson *et al.*, 1990b), the high concentration of circulating 17ß-oestradiol in laying hens may serve physiologically to raise the content of hypothalamic GnRH-I in readiness for the large discharge of GnRH-I and therefore the preovulatory surge of plasma LH.

3.3.1.2 Sex differences in pituitary LH content and concentration

The sex difference in pituitary LH in adults is the result of a reduction in the concentration of pituitary LH in hens rather than an increase in cockerels. This is indicated by the lower concentration of pituitary LH in laying hens than the concentrations in pituitary glands from adult cockerels, juvenile males and juvenile females. These maturational changes may be associated with the increases in the concentration of gonadal steroids in plasma. However, the high concentration of testosterone in adult cockerels is associated with an increase rather than decreases in the maximum LH response to GnRH-I and the concentration of pituitary LH when compared with juvenile cockerels in which concentrations of plasma testosterone are low. This further supports the view that the increase in concentration of plasma 17ß-oestradiol in hens at the onset of puberty is important in the development of the sexually differentiated content of pituitary LH.

A decrease in pituitary LH content in the hen after the onset of sexual maturation might reflect a change in the balance between the rates of LH secretion and LH synthesis. Injections of oestradiol benzoate increase the content of GnRH-I in the hypothalamus of juvenile chickens (Wilson *et al.*, 1990b) and reduce the content of pituitary LHß-mRNA (Kallmeier *et al.*, 1991). The inhibitory feedback effect of 17ß-oestradiol may therefore reduce the release of GnRH-I from the hypothalamus to decrease the stimulatory input to the gonadotroph cells, or act directly on the gonadotroph cells themselves. These observations are consistent with a hypothalamic site of action for 17ß-oestradiol, although a direct action on the pituitary gland is also indicated (see SECTION 3.3.1.3).

The sex difference in pituitary LH content could be due to a lower proportion of LH-containing gonadotroph cells in the pituitary gland of laying hens than in the pituitary gland of adult cockerels (see CHAPTER 4).

3.3.1.3 The role of 17ß-oestradiol in establishing the sex differences in the hormones of the hypothalamic-gonadotroph axis

The increasing concentrations of gonadal steroids in plasma during sexual maturation of the chicken is associated with a decrease in the baseline concentration of plasma LH in hens but an increase in cockerels. This could result from the weaker inhibitory feedback action of testosterone on LH release in cockerels than 17ß-oestradiol in hens (Massa & Sharp, 1985). Thus, the sexually differentiated concentration of plasma LH in adult chickens is due to a more potent suppressive effect of 17ß-oestradiol on the hypothalamic-pituitary complex of laying hens than of testosterone on the hypothalamic-pituitary complex of adult cockerels.

Measurement of GnRH-I content has been used as a marker of how gonadal steroids influence GnRH-I neuronal activity (SECTION 1.3). The greatest difference between the concentrations of GnRH-I between the adult cockerel and laying hen was in the MBH. This area of the brain contains the median eminence, the site of GnRH-I release, and therefore the highest concentration of GnRH-I nerve terminals (SECTION 1.3.1). The content of GnRH-I in the posterior hypothalamus of sexually immature chickens is increased by injections of oestradiol benzoate (Wilson *et al.*, 1990b), presumably by suppressing the release of GnRH-I. The observation that the concentration of GnRH-I in the MBH of females is higher than that of cockerels may therefore indicate an oestrogen-induced suppression of GnRH-I release in hens, and consequently a lower release of LH from the pituitary gland. An additional site for 17ß-oestradiol action in the chicken is the anterior pituitary gland to inhibit LH secretion directly (SECTION 1.5.2). Thus, the GnRH-I-stimulated release of LH from cultures of pituitary cells from juvenile chickens is reduced by direct treatment with 17ß-oestradiol (Luck & Scanes, 1980; King *et al.*, 1989).

The combined suppression of hypothalamic and gonadotroph function by 17ß-oestradiol could therefore establish the sexually differentiated baseline concentrations of plasma LH in the adult chicken. However there were no sex differences in the concentrations of GnRH-I in the MBH and POA, and the concentration of pituitary LH between juvenile cockerels and hens. This observation seems inconsistent with the sexually differentiated concentrations of plasma 17ß-oestradiol and LH in the juvenile chickens. This suggests that gross measurements of pituitary LH content and hypothalamic GnRH-I are not appropriate to detect subtle differences in the function of gonadotroph cells and GnRH-I neurones.

Two hypotheses have been proposed to explain the increased activity of the hypothalamic-pituitary-gonadal axis at the onset of sexual maturity. The first is that the hypothalamic-pituitary complex becomes progressively less sensitive to the negative feedback effects of steroids such that

GnRH, and therefore the release of gonadotrophins increase to stimulate gonadal growth (Negro-Vilar *et al.*, 1973; Smith *et al.*, 1977). Alternatively a neural mechanism, which is independent of steroidal control, increases the 'hypothalamic drive' in the presence of increasing concentrations of steroids to stimulate GnRH release and consequently gonadal development (Sharp, 1974a). Both hypotheses account for the reduced ability of testosterone to reverse the increase in plasma LH after castration as birds approach the onset of puberty (Wilson, 1978). This results in an apparent decrease in the sensitivity of the hypothalamic-pituitary complex of cockerels to the feedback action of testosterone after sexual maturity.

3.3.2 Sex differences in the magnitude of the LH response to GnRH-I

The sexually differentiated magnitude of LH secretion in response to GnRH-I appears to depend on the ability of the pituitary gland to release LH. This ability could be determined by the concentration of LH in the pituitary gland or the potential of the pituitary gland to lose its responsiveness to GnRH-I. The content of pituitary LH in rats is sexually differentiated, and the magnitude of pituitary responsiveness to GnRH is related to the total content of LH (see SECTION 1.5.1.3). The lower content of LH in pituitary glands from laying hens than from those of adult cockerels may account for the smaller increases in plasma LH in laying hens in response to GnRH-I (ΔLH and ΔAUC of LH) than in adult cockerels.

However similar concentrations of pituitary LH were found in juvenile males and females but the maximum responsiveness to GnRH-I (ΔLH) was different. As suggested earlier, this may be because measurement of total pituitary LH is a poor indicator of pituitary function, or that there could be a sex difference in juveniles with respect to the stores of pituitary LH available for immediate release (see CHAPTER 4). A supramaximal dose of GnRH-I was administered to juvenile chickens to estimate this store of 'releaseable' LH. However, the ΔAUC of LH in juvenile males and females was similar. Consequently, other explanations should be considered.

The sex difference in magnitude of ΔLH stimulated by GnRH-I in juvenile chickens could be due to a sex difference in the time-course of LH secretion from the pituitary gland. The rate of decline of the falling phase of plasma LH in juvenile hens was significantly slower than the rate in juvenile cockerels (discussed in more detail in SECTION 3.3.4). This means that although GnRH-I stimulates a smaller ΔLH in juvenile hens, the slower decline in the concentration of plasma LH compensates for this difference to produce an equivalent ΔAUC of LH to the juvenile cockerel. This would produce a similar total ΔAUC of LH to be achieved between juvenile males and females and allow a sex difference in the peak concentration of ΔLH.

Alternatively, there may be a sex difference in the rate of loss of pituitary responsiveness to GnRH-I. A more rapid development of desensitisation or depletion of LH in pituitary glands of juvenile cockerels than in juvenile hens would produce the more rapid initial decrease in plasma LH observed in males than in females. Consequently, although the ΔLH response of juvenile

cockerels to GnRH-I was larger than that of juvenile females, the male pituitary gland may lose its responsiveness to GnRH-I more rapidly, and therefore secrete less LH during the remaining period of the response to produce the similar ΔAUCs of LH for juvenile males and females.

The sex difference in the resting concentration of LH in adult chickens and the magnitude of LH release may be due at least in part to age or sex-related differences in the immunoreactive properties of the endogenous LH of adult cockerels and laying hens. The chicken LH antiserum used in the radioimmunoassay to measure plasma LH (anti-LH-3/3; Sharp *et al.*, 1987) was raised against a purified preparation of LH (PRC-AE1-1) isolated from pituitary glands of juvenile chickens of both sexes. This fraction contains four peaks of LH immunoreactivity which probably represent different isoforms of LH (Talbot *et al.*, 1988). GnRH, inhibin, steroids and the age, sex and reproductive status of mammals influence the gene expression of α-subunit, LHß and FSHß subunits, and the post-translational processing of these polypeptides (reviewed by Wilson *et al.*, 1990d). Consequently the secreted forms of LH in adult chickens may undergo glycosylation or sialylation to different extents and therefore differ immunoreactively from those forms in juvenile chickens against which the LH-antibody was originally raised. However, the LH radioimmunoassay was able to detect sex differences in the LH responses of 7.5-week old juvenile male and female chickens. This means that any differential cross-reactivity of anti-LH-3/3 between the adult male and female forms of LH may not be significant, and the features of the sex difference to GnRH-I in adult chickens would therefore be genuine.

3.3.3 Sex difference in the pituitary sensitivity to GnRH-I

The release of LH was increased by GnRH-I in a dose-dependent fashion in adult cockerels, but not in laying hens. There is also a dose-response relationship between GnRH-I and the release of LH in juvenile chickens of both sex (Wilson *et al.*, 1989; Nakamura *et al.*, 1991). Ten-times more GnRH-I was required to stimulate a maximum release of LH in laying hens than in adult cockerels. It has been observed that the maturational reduction in pituitary sensitivity to mGnRH is correlated with the increase in concentration of plasma 17ß-oestradiol (Bonney *et al.*, 1974; Wilson & Sharp, 1975b; Knight *et al.*, 1985). These observations could be due to fewer GnRH receptors, or receptors with a lower affinity for GnRH-I in the pituitary gland of laying hens, than in pituitary glands of adult cockerels. The high concentration of 17ß-oestradiol in laying hens may induce these differences, however it is uncertain whether 17ß-oestradiol acts on the pituitary gland directly or indirectly through a decrease in GnRH-I release from the hypothalamus. Unfortunately, previous attempts to measure these receptors have not been successful and therefore this possibility cannot be ruled out (see CHAPTER 4).

3.3.4 Sex differences in the time-course of GnRH-I-stimulated LH release

The longer time required for the GnRH-I-stimulated concentration of LH to return to baseline in laying hens compared with adult cockerels, confirms the sex difference in duration of the LH

response stimulated by intravenous injections of GnRH-I (Sharp *et al.*, 1987) and subcutaneous injections of mGnRH or GnRH superagonists (Sterling & Sharp, 1984). There was no difference in the rate of disappearance of ^{125}I-LH from plasma (FIGURE 3.4). This means that the prolonged response of the laying hen is not due to a sex difference in the efficiency of the mechanisms involved in the catabolism of the injected LH. Consequently, since the concentration of plasma LH is the product of LH secretion and LH clearance processes, this suggests that the difference in duration of elevated LH could be related to the rate of LH *secretion* from the pituitary gland. However it is difficult to make a careful study of GnRH-I-stimulated LH secretion from the pituitary gland *in vivo* due to the plasma half-life effects of injected GnRH-I and of LH, and the interactions of the hypothalamic-gonadotroph-gonadal axis (CHAPTER 1). This type of analysis requires an *in vitro* system to study the pituitary gland in isolation (see CHAPTER 4).

The sex difference in duration of elevated LH in response to GnRH-I could also be interpreted as a shorter response in adult cockerels than in laying hens due to loss of responsiveness of the pituitary gland to GnRH-I. This would reduce the release of LH in response to GnRH-I and allow the concentration of plasma LH to decrease. The development of desensitisation to GnRH-I or mGnRH has been studied in isolated pituitary tissue from chickens (King *et al.*, 1987, 1988) and turkeys (Guémené & Williams, 1992a). In these *in vitro* experiments, a previous exposure to the releasing hormone reduces the size of the LH response to a second exposure to the peptide. In contrast, the induction of loss of pituitary responsiveness to GnRH in laying hens requires several days of sustained stimulation with high doses of GnRH analogues (Dickerman & Bahr, 1989; Tilbrook *et al.*, 1992). This decrease in responsiveness could represent either desensitisation to GnRH-I or depletion of the pituitary stores of LH. The ability of GnRH-I to induce loss of responsiveness of the pituitary gland in adult chickens may therefore account for the sex difference in duration of elevated LH.

Alternatively, the pituitary gland of laying hens but not of adult cockerels may continue to release LH after the concentration of GnRH-I in plasma has declined to levels insufficient to stimulate LH secretion.

The sex difference in sensitivity to GnRH-I (Sharp *et al.*, 1987; SECTION 3.3.3) suggests that more GnRH-I must be released in laying hens than in adult cockerels to increase plasma LH. Consequently, although the plasma half-life of GnRH-I in both of the adult sexes is 3-minutes (Sharp *et al.*, 1987), the larger dose of GnRH-I given to laying hens may circulate for longer and therefore prolong the duration of elevated LH compared with adult cockerels. However this sex difference was still observed after the administration of the same dose of GnRH-I (20 µg GnRH-I/kg) to adult cockerels and laying hens. This suggests that the difference in the duration of elevated LH is not due to the different doses of GnRH-I given to adult cockerels and laying hens in the study of Sharp *et al.* (1987).

Another explanation which might account for these observations derives from reports that injections of very large doses of mGnRH or GnRH superagonists in laying hens elicit two phases of elevated LH, whereas moderate doses produce a single prolonged phase of LH secretion (Sterling & Sharp, 1984; Guémené & Williams, 1986). The dose of 20 μg GnRH-I/kg given to adult hens in the present study is within the range of these 'moderate' doses of GnRH. A biphasic LH response is also seen during constant infusion of GnRH in men and women (Bremner & Paulsen, 1974; Hoff *et al.*, 1977). This effect may be mediated through stimulation of LH synthesis in the pituitary gland which is later released during the second phase of secretion, and two pools of pituitary LH have been proposed to explain these observations. The first phase of release originates from a 'readily releaseable' pool of LH whereas the later phase derives from either a 'storage' pool of LH or a newly synthesised pool of LH (Bremner & Paulsen, 1974; Hoff *et al.*, 1977). A similar series of events could explain the observations in laying hens.

A progesterone-dependent mechanism (Guémené & Williams, 1986) may also explain the prolonged duration of elevated LH in laying hens injected with a moderate dose of GnRH-I. The spontaneous preovulatory surge of LH develops through the release of GnRH-I from the hypothalamus (see SECTION 1.3.3) which coincides temporally with increases in plasma progesterone by 1 - 3 ng/ml (Furr *et al.*, 1973a; Etches & Cunningham, 1976; Williams & Sharp, 1978b; Etches & Cheng, 1981). Similar increases in the concentration of plasma progesterone are achieved after injections of either ovine LH given 12-hours (Shahabi *et al.*, 1975a) or 4.5 - 9-hours (Etches *et al.*, 1983) after ovulation, or high doses of mGnRH or its analogues given 4 - 9-hours after ovulation (Guémené & Williams, 1986). These increases of progesterone therefore occur in the absence of a ripe preovulatory follicle. Since a similar time of GnRH-I injection was used in the present work (3 - 5-hours after the estimated time of ovulation) it is possible that GnRH-I produces the extended duration of plasma LH in laying hens through increasing the release of progesterone. GnRH itself also synergises with LH to directly increase progesterone production from cultures of ovarian granulosa cells of the chicken (Culbert *et al.*, 1980; Hertelendy *et al.*, 1982). The stimulatory action of progesterone would not be maintained indefinitely however, and the concentration of LH eventually returns to baseline. The termination of this action of progesterone may be due to the immaturity of the preovulatory follicle and its relatively low ability to secrete progesterone compared with a mature follicle (Shahabi *et al.*, 1975b). This also explains the absence of a surge of LH in response to injection of GnRH-I in the present observations in laying hens. A similar prolonged LH response after GnRH-I injection is not seen in adult cockerels and juvenile chickens because a fully developed ovary is not present. Alternatively, a sustained stimulation of the pituitary gland by GnRH-I depletes the acutely releaseable store of LH. This is consistent with the report that the falling phase of the preovulatory LH surge is associated with a reduced pituitary responsiveness to GnRH-I (Wilson *et al.*, 1990a), and the observation that progesterone directly reduces the responsiveness of pituitary cells from juvenile chickens to GnRH-I (King *et al.*, 1989).

The rates of decline of the falling phase of LH in juvenile chickens injected with GnRH-I were faster than those for both sex of adults, and faster than the plasma half-life of ^{125}I-LH in adult birds. This could mean that LH is cleared from plasma more rapidly or that the secretion of LH from the pituitary gland ends more quickly in juveniles than in adults. Alternatively, there may be differences in the half-lives of the endogenous LH from adult and juvenile chickens.

In mammals, the proportions of LH isoforms and the circulatory half-life of LH are influenced strongly by the concentrations of GnRH, testosterone and 17ß-oestradiol in plasma. Adult chickens were injected with a preparation of LH obtained from juvenile chickens of both sexes (PRC-AE1-s-1; Sharp *et al.*, 1987). However the plasma half-life of LH in humans and rats depends on the degree of glycosylation and sialylation of the LH isoforms, and this is affected by testosterone, 17ß-oestradiol and GnRH (reviewed by Wilson *et al.*, 1990d). This could mean that the proportions of LH isoforms in adult chickens differ from those of juvenile chickens. In this respect, the extent of sialylation of the LH isoforms is respectively increased or decreased by testosterone and 17ß-oestradiol, which produce mutually opposing effects on the circulatory half-life of LH. These effects of steroids are due to changes in the relative proportions of the LH isoforms. Oestrogen also has the ability, as does GnRH, to promote glycosylation of LH to lower the half-life of LH in plasma. Thus, sialylation and glycosylation of LH produce more acidic or more basic forms of LH respectively. It is not surprising therefore that the isoforms of LH and FSH in the pituitary gland of adult female rats differ in proportions and are less acidic than those of the adult male (Wakabayashi, 1980; Foulds & Robertson, 1983; Hattori *et al.*, 1983; Blum *et al.*, 1985). Consequently, the circulatory half-life of the less acidic forms of LH from female rats is shorter than those forms from males. This means that testosterone and 17ß-oestradiol regulate the life-span of LH in plasma.

The observation that the plasma half-life of injected ^{125}I-chicken-LH into birds is unaffected by the sexual maturity of quail (Scanes & Follett, 1973) or by gonadectomy of hens and cockerels (Wilson, 1975), does not rule out an effect of gonadal steroids on the half-life of LH in chickens. This is because the technique for evaluating half-life actually measures the *efficiency* of the mechanisms for removing purified LH (which originates from pituitary glands of juvenile chickens) from plasma, rather than the actual half-life of *endogenous* LH in plasma. It remains to be seen whether LH derived from pituitary glands of laying hens has a longer half-life in plasma than that from adult cockerels. Also, it is not known whether testosterone and 17ß-oestradiol affect the post-translational processing of LH and therefore the plasma half-life of LH, or whether the relative proportions of LH isoforms are sexually differentiated in the adult chicken.

3.3.5 Sex difference in the control of baseline concentrations of plasma LH by GnRH-I

The baseline concentration of plasma LH in adult chickens appears to be sexually differentiated with respect to its dependency on GnRH-I release. This was indicated by the observation that

treatment with a single dose of anti-GnRH-I serum reduced the concentration of plasma LH in adult cockerels but not in laying hens. GnRH-I can regulate the release of LH from gonadotroph cells directly by stimulating LH secretion, and indirectly by stimulating LH synthesis thereby increasing the 'spontaneous' release of LH (CHAPTER 1). Consequently the baseline concentration of LH in adult cockerels is in part directly dependent on GnRH-I release, whereas the resting level of LH in laying hens is indirectly dependent on GnRH-I release. The reduction in plasma LH in the cockerel after passive immunisation with anti-GnRH-I serum is consistent with the episodic pattern of GnRH-I release from the hypothalamus required to maintain the concentration of plasma LH (see SECTION 1.3.4). Furthermore, the absence of a detectable episodic pattern of plasma LH in laying hens (Wilson & Sharp, 1975c) suggests that, outside the preovulatory surge of LH, the release of GnRH-I may not control the baseline levels of LH in the same way as in cockerels.

A component of LH release in adult cockerels also appears to be indirectly dependent on GnRH-I release. This is suggested by the incomplete and transient suppression of plasma LH by anti-GnRH-I, which occured despite sufficient peaks levels of anti-GnRH-I to theoretically bind 1050 pg GnRH-I/ml of plasma. No information is available on the portal blood concentrations of GnRH-I arriving at the pituitary gland of the chicken, but deductions can be made from observations in other species. For example, concentrations of 2 - 250 pg GnRH/ml occur in the portal blood of rats (Sarkar, 1987; Phelps *et al.*, 1992), 17 - 36 pg/ml in the ewe (Clarke & Cummins, 1982; Caraty *et al.*, 1989), and up to 104 pg/ml in the female monkey during the preovulatory LH surge (Neill *et al.*, 1977). The concentration of anti-GnRH-I measured after passive immunisation of cockerels, binds at least four-times the highest reported values of GnRH in mammalian portal blood (250 pg/ml; Sarkar, 1987; Phelps *et al.*, 1992). However in addition to antibody concentration, the affinity of the antibody for GnRH-I will also affect its potential for immunoneutralising GnRH-I. The affinity of the antiserum for GnRH-I was not determined.

The concentration of GnRH-I in the portal blood vessels supplying the anterior pituitary gland of the adult cockerel can be estimated from a number of observations. The concentration of plasma LH in the adult cockerel exhibits pulsatility (Wilson & Sharp, 1975c) which probably reflects a pulsatile pattern of GnRH-I secretion from the hypothalamus (see SECTION 1.3.4). These pulses of plasma LH represent increases of between 1 - 11 ng/ml (Wilson & Sharp, 1975c). Injection of a low dose of 0.1 μg GnRH-I/kg in a 2.8 kg adult cockerel increased the concentration of plasma LH to within this range by 3.7 ± 1.0 ng/ml (TABLE 3.5). This small rise in plasma LH was achieved by an estimated concentration of 2.1 ng GnRH-I/ml of plasma (assuming a plasma volume of 4.7 ml/100 g body weight in adult cockerels; Sturkie, 1986). This concentration of GnRH-I is double the theoretical binding capacity calculated for the maximum circulating level of anti-GnRH-I in the cockerel. It is therefore possible that this explains the partial and transient suppression of plasma LH by anti-GnRH-I.

A similar calculation can be made for the concentration of plasma GnRH-I required to stimulate LH release from the pituitary gland of the laying hen. GnRH-I and mGnRH are equipotent in their ability to release LH (compare Sterling & Sharp, 1984 and Sharp *et al.*, 1987). Injection of 1 μg mGnRH/kg does not increase the concentration of plasma LH in laying hens (Sterling & Sharp, 1984; Guémené & Williams, 1986) but 5 μg GnRH-I/kg was sufficient to increase the concentration of plasma LH in the present study (TABLE 3.5). Assuming a plasma volume of 4.7 ml/100g body weight (Sturkie, 1986) in a 1.8 kg laying hen, 1 and 5 μg GnRH-I/kg produce respective concentrations of 21 and 106 ng GnRH-I/ml of plasma. These concentrations of GnRH-I exceed the maximum binding capacity of anti-GnRH-I in plasma (1.25 ng GnRH-I/ml) by 17 - 85-times. However, only 0.58 ng GnRH-I/ml is necessary to stimulate LH release directly from pituitary cells of the laying hen (Wilson *et al.*, 1990a). A 2-fold excess of anti-GnRH-I was achieved in laying hens to block this LH-releasing activity. No *in vitro* studies were made to confirm this. The conflict between the calculations made *in vivo* and those *in vitro* make it difficult to establish whether sufficient anti-GnRH-I was present in plasma to neutralise the concentration of GnRH-I in portal blood. However, the *in vitro* study of Wilson *et al.* (1990a) using pituitary cells stimulated directly with GnRH-I provides a more reliable source of information than that from estimations derived from *in vivo* data. It is therefore likely that adequate levels of anti-GnRH-I were achieved in the plasma of laying hens to prevent the action of GnRH-I on plasma LH.

The possibility that LH release in the chicken may be partly independent of GnRH-I release is supported by studies in short and long-term castrated rats. These studies show differential effects of treatments which modify the action of GnRH on the concentration of plasma LH (Almeida *et al.*, 1989). Thus in contrast with short-term castrates, the elevated concentration of plasma LH in long-term castrates is resistant to reversal by GnRH-receptor antagonists, morphine, immunoneutralisation of GnRH, and the blockade of neuronal transport of GnRH (and therefore GnRH release). This GnRH-independent high concentration of plasma LH indicates some autonomy of LH synthesis and release by the anterior pituitary gland (Almeida *et al.*, 1989). Furthermore, the elevated concentration of plasma LH in long-term ovariectomised females but not long-term castrated males is reversed by a GnRH-receptor antagonist (Almeida *et al.*, 1988). This appears to reveal a sex difference in the control of LH release by GnRH in rats at the level of the pituitary gland. Similar studies to these have not been made in the adult chicken. However the passive immunisation experiment here suggests a sex difference in the role of GnRH-I in the regulation of basal LH release from the anterior pituitary gland.

There are two possibilities for a GnRH-I-independent control of plasma LH. The first is that the pituitary gland has an inherent basal output of LH, and the secondly that plasma LH is regulated by some releasing factor other than GnRH-I. Although the anti-GnRH-I serum does not cross-react with GnRH-II (PJ Sharp, personal communication), it is unlikely that GnRH-II is important in the direct control of LH because of the absence of GnRH-II nerve terminals in the median eminence (SECTION 1.3.1). Another explanation for the inability of the anti-GnRH-I serum to inhibit completely the concentration of plasma LH in the chicken could be due to cross-reactions of the

LH-antibody in the LH radioimmunoassay, with chicken thyroid-stimulating hormone (TSH) and FSH (Follett *et al.*, 1972; Sharp *et al.*, 1979, 1987). Consequently, the apparent 'baseline' concentration of plasma LH would represent the net concentrations of TSH, FSH and LH. Only the extent to which TSH cross-reacts in the LH assay is unknown due to the inavailability of a satisfactorily pure preparation of chicken TSH and consequently the lack of a specific assay for chicken TSH. The cross-reaction of chicken FSH in the LH assay has a fractional potency of only 0.002 relative to chicken LH (Krishnan *et al.*, 1992). Thus the possibility cannot be excluded that the concentration of TSH in plasma contributes significantly to the baseline concentration of LH-immunoreactivity in the chicken.

Alternative reasons for the incomplete suppression of plasma LH by anti-GnRH-I in cockerels are that the antiserum is rapidly cleared from the circulation or that a compensatory mechanism counters the fall in LH concentration. Normal concentrations of LH were restored by 24-hours after injection of anti-GnRH-I, and accompanied by a downward trend in the levels of anti-GnRH-I in plasma. However a sufficient antibody concentration remained after 24-hours to bind 569 pg GnRH-I/ml of plasma. This level still exceeds the portal concentrations of GnRH in mammals by 2-fold, but is sufficient to bind only a quarter of the calculated level of circulating GnRH-I in cockerels.

Passive immunisation against mammalian GnRH (Fraser & Sharp, 1978) or active immunisation against GnRH-I (Sharp *et al.*, 1990) reduce the concentration of plasma LH in laying hens. This contrasts with the present results and may suggest that insufficient anti-GnRH-I serum (adequate to bind 1250 pg GnRH-I/ml) was used in the present experiments to neutralise the circulating levels of GnRH-I in adult hens. A subsequent experiment increased the anti-GnRH-I concentration to levels sufficient to bind 6 - 10-times the maximum mammalian concentrations of plasma GnRH, or 3 - 4-times the concentration of GnRH-I required to stimulate LH release from pituitary cells from laying hens. This treatment reduced the magnitude of the preovulatory LH surge and the concentration of LH at the end of the experiment, but did not block egg-laying. This means that the absolute concentration of LH achieved during the surge is not critical for ovulation. A similar conclusion was reached from observations that pharmacological manipulation of the neurotransmitters involved in GnRH release from the hypothalamus, markedly reduced the preovulatory LH surge, but almost all of the hens ovulated normally (Knight *et al.*, 1982b).

The physiologically important concentration of GnRH-I is that present in the portal blood system. Considering the low sensitivity of the pituitary gland of the laying hen to GnRH-I compared with that of the cockerel (Sharp *et al.*, 1987), there must be a substantial release of GnRH-I from the hypothalamus to induce a preovulatory surge of LH. This is suggested by the 50% reduction in GnRH content in the hypothalamus which coincides with the natural preovulatory surge of LH (Knight *et al.*, 1984). It is possible therefore, that such a large discharge of GnRH-I into the portal system exceeded the neutralising capacity of anti-GnRH-I in the present experiments to result in a pituitary stimulation sufficient to evoke an LH surge and an ovulation. The discrepancy between

the present observations and the success of a previous study to block both the progesterone-induced LH surge and egg-laying (Fraser & Sharp, 1978) may be due to the difference between studying the spontaneous LH surge here, and the progesterone-induced surge of LH. This would indicate that whereas progesterone elevates the concentration of LH by inducing the release of GnRH-I, the natural LH surge may involve GnRH-I *and* some other factor. More definitive studies are needed to validate this possibility.

The partial neutralisation of the 'preovulatory release of GnRH-I' to reduce the amplitude of the LH surge, may also deprive the pituitary gland of sufficient GnRH-I to allow adequate synthesis of LH to replenish the stores of pituitary LH. Thus the reduction in the baseline concentration of LH at the end of this experiment may have arisen because of depleted stores of LH.

It is clear that the anti-GnRH-I serum used here revealed a sex difference in the control of baseline concentrations of LH by GnRH-I in adult chickens. However it is not certain whether a complete neutralisation of GnRH-I in plasma was achieved. An alternative approach to compare the relative importance of GnRH-I in the control of the concentration of plasma LH in adult chickens might be to use a GnRH-receptor antagonist to prevent the action of GnRH at the pituitary gland.

3.4 Summary

The pituitary gland of the laying hen *in vivo* was less sensitive and less responsive to GnRH-I, and the time-course of LH released was more prolonged compared with the LH response of the adult cockerel. GnRH-I released similar amounts of LH in juvenile males and females with a similar time required to reach peak concentrations of LH. The magnitude of LH release by GnRH-I (ΔLH and ΔAUC) may depend on the concentration of LH in the pituitary gland. On reaching sexual maturity, the concentration of pituitary LH decreased in hens but remained the same in cockerels. The sex difference in time-course of the GnRH-I-stimulated release of LH from the adult but not the juvenile chicken, could be due to differences in the rate of LH secretion from the pituitary gland, rather than a difference in the efficiency of the mechanisms for clearing LH from plasma. This may be due to differences in the susceptibility of the pituitary gland to desensitisation by GnRH-I, or the presence of a stimulatory effect of progesterone in the laying hen, or a sex difference in the circulatory half-life of endogenous LH in adult chickens. The baseline concentration of LH was sexually differentiated in adult chickens and to a lesser extent in the juvenile chicken. This may be due to the higher concentration of plasma 17ß-oestradiol, and therefore greater inhibitory feedback on the pituitary gland and/or the hypothalamus, in hens than in cockerels at both ages. Passive immunisation against GnRH-I revealed a sex difference in the dependency on GnRH-I release in the maintenance of baseline levels of LH in the adult cockerel and hen. This could suggest that the regulation of LH synthesis and release by GnRH-I in the pituitary gland of the adult chicken is sexually differentiated.

Studies on the concentrations of GnRH-I and GnRH-II in the hypothalamus suggested no physiological role for GnRH-II in the cockerel. However, GnRH-II may be important in laying hens by interacting with GnRH-I neurones to regulate reproductive function. There were maturational increases in the concentrations of hypothalamic GnRH-I and these were sexually differentiated in adult but not juvenile chickens. The higher concentration of GnRH-I in the MBH of laying hens compared with that of the adult cockerel, probably represents accumulation of GnRH-I through sustained suppression of its release by 17ß-oestradiol.

The development of the sexually differentiated LH responses to GnRH-I injection in adult chickens is probably due to the maturational increase in the concentration of plasma 17ß-oestradiol in the hen rather than of testosterone in the cockerel.

4.1 INTRODUCTION

In the previous chapter it was shown that there are sex differences in the sensitivity, magnitude and profile of LH release in response to an intravenous injection of GnRH-I which are pronounced in adult chickens but less so in juveniles. The pituitary response to GnRH-I is influenced by hormonal interactions of the hypothalamic-pituitary-gonadal axis (CHAPTER 1), and the effects of metabolism and clearance of LH (CHAPTER 3). The sex differences on LH secretion may be mediated by gonadal steroids acting primarily on the anterior pituitary gland to reduce its responsiveness and sensitivity to GnRH-I. This could be achieved by affecting GnRH-receptors, signal transduction, or the synthesis and release of LH. It could also act directly on the hypothalamus to reduce GnRH-I release which in turn could reduce the synthesis and release of LH by the anterior pituitary gland. The extrapituitary effects of 17ß-oestradiol and the effects of LH clearance from plasma can be excluded by investigating the release of LH from the isolated pituitary gland. The use of an *in vitro* system allows a focussed study to be made on the control of LH secretion by GnRH-I in a defined environment. A detailed *in vitro* investigation was made of the LH responses of pituitary glands from adult cockerels and laying hens to GnRH-I. Comparative studies using pituitary glands from juvenile chickens were also carried out to establish whether the LH responses to GnRH-I are sexually differentiated *in vitro*. These experiments sought to characterise the components of the sexually differentiated LH response to GnRH-I, and to identify possible causes.

4.2 RESULTS

4.2.1 Static incubations of pituitary glands

4.2.1.1 Sex differences in the 'readily releaseable' pool of LH in the pituitary gland

In CHAPTER 3, it was suggested that the sexually differentiated responsiveness of the pituitary gland to GnRH-I are related to the sexually differentiated concentration of pituitary LH. A large content of pituitary LH does not necessarily correlate with a high LH secretory capacity because not all the LH is available for immediate release. Of more *physiological* relevance is a pool of LH which lies immediately beneath the plasma membrane, the 'readily releaseable' pool of LH (RRP; Bremner & Paulsen, 1974; Hoff *et al.*, 1977; Adams & Nett, 1979; Pickering & Fink, 1979;

Lewis *et al.*, 1985, 1986; Gracia-Navarro *et al.*, 1990). Owing to the sex difference in sensitivity to GnRH-I (Sharp *et al.*, 1987) and to avoid the development of desensitisation (de Koning *et al.*, 1978; Zilberstein *et al.*, 1983; King *et al.*, 1986, 1987; Guémené & Williams, 1992a), a Ringer's solution containing a high concentration of K^+ rather than GnRH-I was used to stimulate LH release from pituitary tissue.

The baseline release of LH and the K^+-releaseable pool ('readily releaseable' pool, RRP) of LH from pituitary glands of laying hens were lower ($P<0.001$; TABLE 4.1) than those from pituitary glands of adult cockerels. The RRP of LH represents 22% of the total content of pituitary LH in adult cockerels (TABLE 3.2) and 19% of pituitary LH in laying hens. The basal and RRP of LH between the 7.5-week old sexes were not statistically different, representing 8 and 6% of total pituitary LH (TABLE 3.2) in the male and female respectively.

TABLE 4.1: Comparison of the basal release and 'readily releaseable' stores of LH in pituitary glands of adult and juvenile chickens *in vitro*.

		Basal LH (ng/mg tissue)	RRP (ng/mg tissue)
ADULT	**Male**	32.4 ± 3.2	212.5 ± 42.5
	Female	**22.8 ± 2.7	***30.9 ± 3.0
JUVENILE	**Male**	26.7 ± 2.1	68.5 ± 4.2
	Female	^{NS}24.9 ± 3.8	^{NS}68.5 ± 8.2

Basal and K^+-stimulated release of LH (n = 8 per sex per age) were measured from pituitary tissues incubated for 30-min in respectively Avian Ringer, and Avian Ringer containing 60 mM K^+. Adults and juveniles were respectively, 21 and 7.5-weeks-old. Readily releaseable pool of LH (RRP), LH_{KCl} - LH_{BASAL}. LH release was corrected for pituitary weight (TABLE 3.2). NS = not significantly different, **P<0.01, ***P<0.001 compared with age-matched male.

4.2.1.2 Sex difference in the LH response to GnRH-I in static incubations of juvenile and adult pituitary tissues

A dose-response relationship between GnRH-I concentration and LH release was established for pituitary glands from juvenile cockerels and hens, with ED_{50} values of 5 and 2 nM respectively, and a maximal release of 5.3 and 5.7-times the unstimulated release of LH (not significantly different from each other; FIGURE 4.1).

GnRH-I also increased the release of LH from pituitary tissue from adult cockerels and laying hens (FIGURE 4.2). Pituitary tissue from males were more responsive than that from laying hens ($P<0.001$). ED_{50} values for GnRH-I of 9 nM and 30 nM were estimated for pituitary glands of adult cockerels and hens respectively.

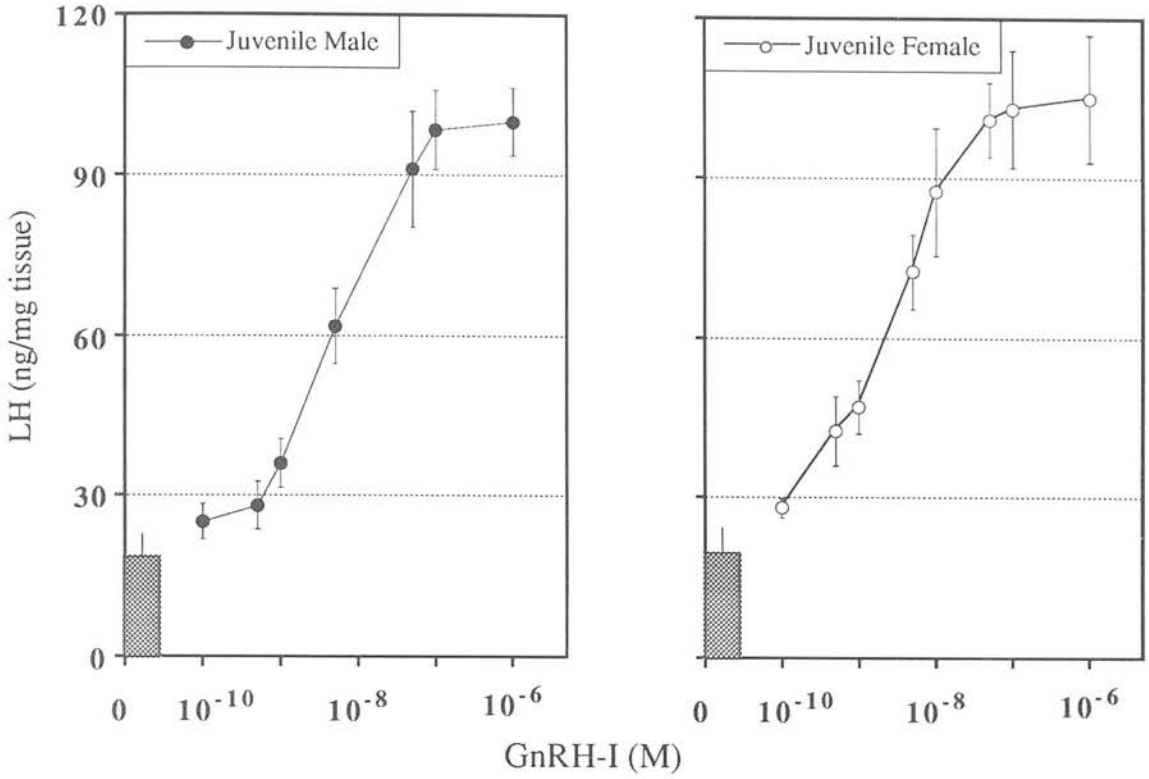


FIGURE 4.1: Dose-response curves for GnRH-I-induced LH release from pituitary glands from juvenile male and female chickens.
Hemipituitary glands from juvenile males and females were incubated in the absence (shaded bar) and presence of 0.1 - 1000 nM GnRH-I for 60-min. Results are expressed as ng LH/mg tissue weight (n = 8).

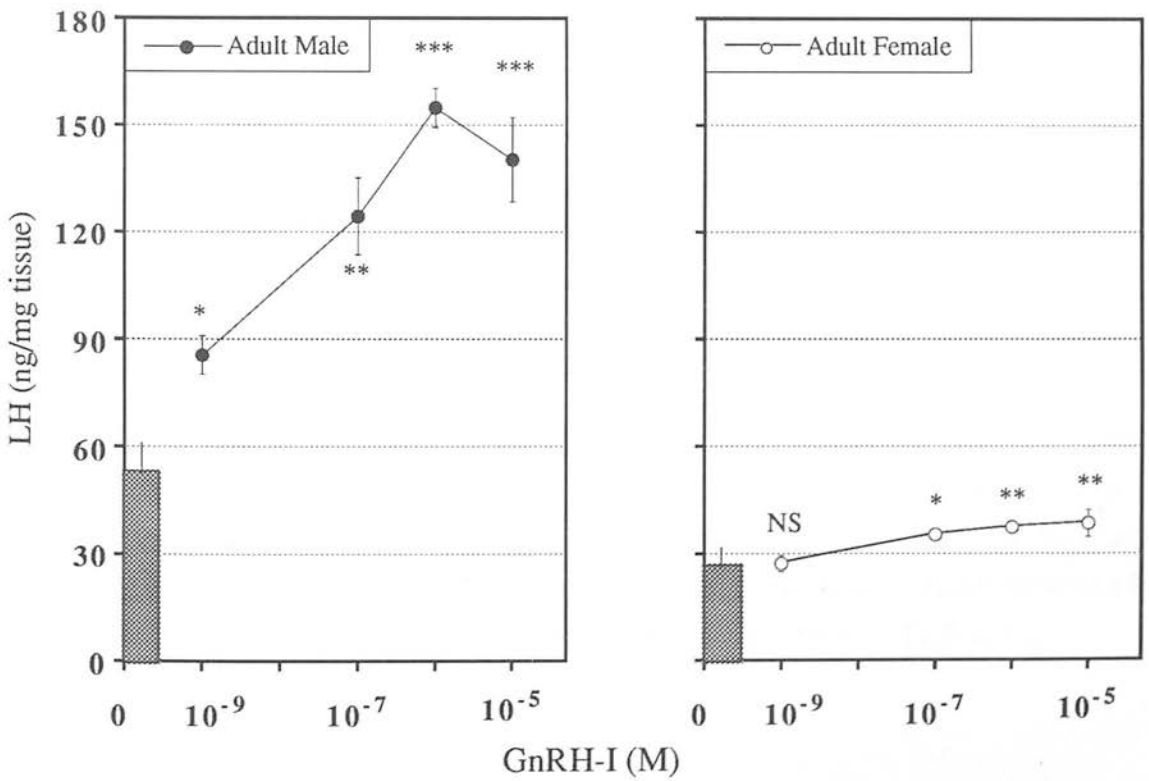


FIGURE 4.2: Dose-response relationship between GnRH-I and LH release from pituitary tissue from adult cockerels and laying hens.
Quartered pituitary glands from male and female adult chickens (n = 12 per dose of GnRH-I) were treated with GnRH-I for 60-min and the release of LH was expressed as ng LH/mg tissue. NS = not significantly different, *P<0.05, **P<0.01, ***P<0.001 compared with basal LH release. Note the different scales on the y-axes.

4.2.2 Sex differences in the dynamics of LH secretion from the pituitary gland

4.2.2.1 Sex differences in the pattern of LH release in response to infusions of GnRH-I

It was shown in CHAPTER 3 *in vivo* that there is a sex difference in the pattern of LH secretion from the pituitary gland of the adult chicken. However the extent to which this difference was due to sex differences in clearance rates of hormones from plasma or interactions of the hypothalamic-pituitary-gonadal axis was not established. The static incubation studies provide information on the responsiveness and sensitivity of the pituitary gland to GnRH-I, but give no indication of the detailed time-course of LH secretion. A perifusion apparatus was used to follow the dynamics of LH release from the pituitary gland in response to GnRH-I.

The baseline release of LH from perifused pituitary tissue from laying hens was significantly lower (1.5 ± 0.2 ng/ml/mg) than the release from pituitary tissue from adult cockerel (3.8 ± 0.2 ng/ml/mg; $P<0.001$, n = 25/30; FIGURE 4.3).

As was observed *in vivo*, the profile of GnRH-I-stimulated LH release from adult pituitary glands was sexually differentiated *in vitro* (FIGURE 4.3). In the continuous presence of GnRH-I, pituitary glands from adult cockerels released LH in two distinct phases whereas pituitary glands from laying hens apparently released LH in a single phase. The male-type response was composed of a transient 'spike' of LH release followed by a 'plateau' phase. The concentration of LH during the plateau phase was sustained while GnRH-I was present and returned to baseline levels within 20-minutes after withdrawal of GnRH-I. An initial spike of LH release from pituitary glands from laying hens was not observed after the beginning of perifusion with GnRH-I. Instead, GnRH-I stimulated a single phase of LH release at a low rate which persisted until the withdrawal of the stimulus, and then decreased to the baseline within 20-minutes.

The baseline release of LH from perifused pituitary tissue from juvenile males (0.7 ± 0.2 ng/ml/mg) was not significantly different from that of juvenile females (0.8 ± 0.3 ng/ml/mg; n = 16 - 20). The profile of GnRH-I-stimulated LH release from pituitary glands of juvenile males and females had similar biphasic profiles (FIGURE 4.3). The spike and plateau concentrations of LH released from pituitary tissues from juvenile males and females are shown in TABLE 4.2.

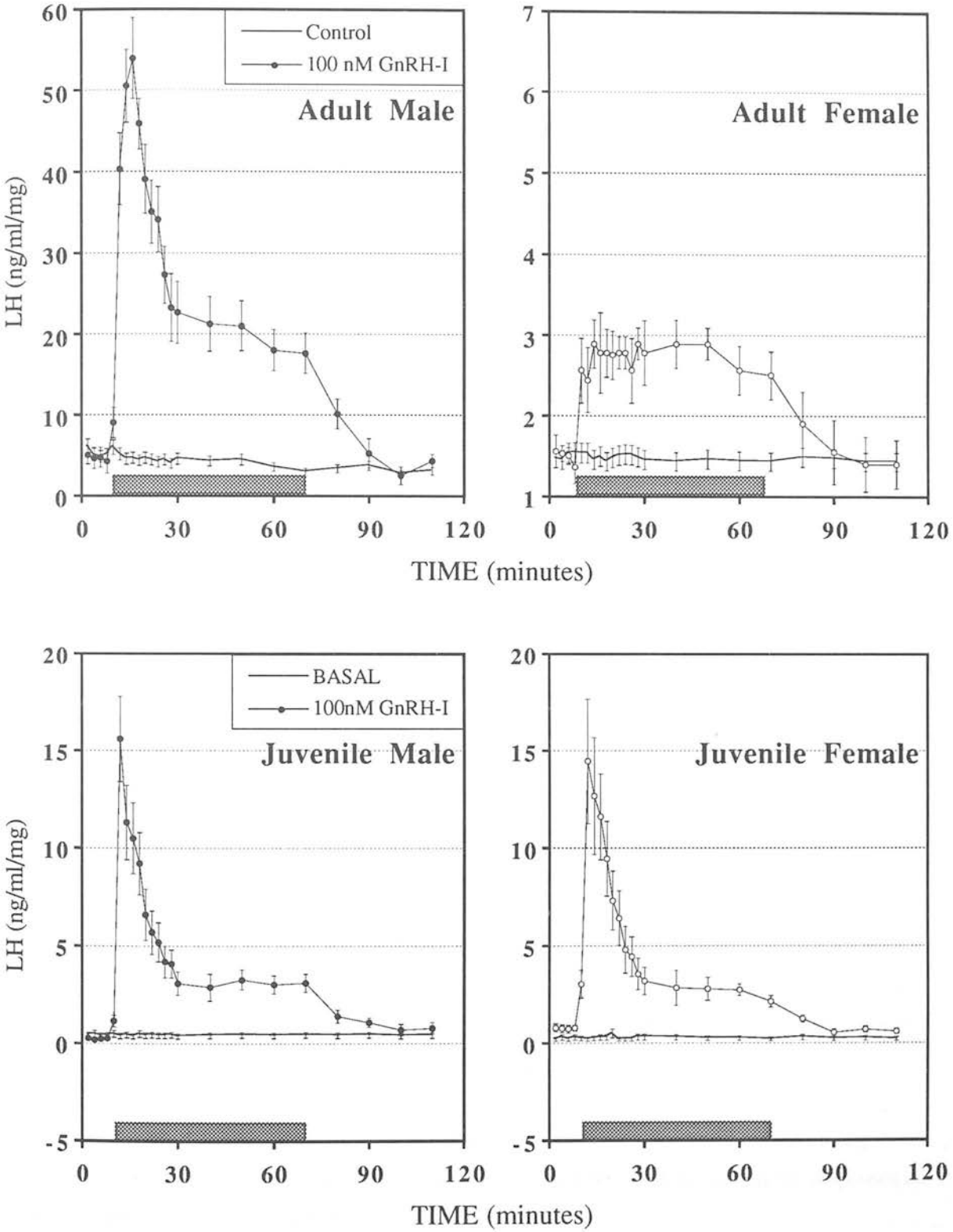


FIGURE 4.3: Sex difference in the profile of GnRH-I-stimulated release of LH from perifused pituitary glands from male and female adult and juvenile chickens.
Quartered pituitary glands from adult and juvenile males and females (n = 4/6 per sex) were perifused with 100 nM GnRH-I for 60-min (grey bar). Perifusions of pituitary tissue from males and females were run in parallel. Two-min fractions of perifusate were collected continuously or at 8-min intervals. Note the different scales on the y-axes.

TABLE 4.2: Sex difference in the effect of GnRH-I on the profile of LH release from perifused pituitary tissue from adult and juvenile cockerels and hens.

		Spike ΔLH (ng/ml/mg)	Plateau ΔLH (ng/ml/mg)
ADULT	**Male**	49.3 ± 5.0	16.3 ± 3.1
	Female	***1.9 ± 0.4	**1.7 ± 0.4
JUVENILE	**Male**	14.5 ± 2.2	2.8 ± 0.8
	Female	^{NS}13.5 ± 3.2	^{NS}1.7 ± 0.4

Spike ΔLH = maximum ΔLH concentration between 0 - 10-min of GnRH-I stimulation. Plateau ΔLH = mean ΔLH concentration between 20 - 70-min of GnRH-I stimulation. NS = not significantly different, **P<0.01, ***P<0.001 compared with the male (n = 4/6).

4.2.2.2 Sex differences in the release of LH after repeated stimulation with GnRH-I

The descending portion of the GnRH-I-stimulated LH spike, to the plateau rate of release may be due to the development of a partial desensitisation to GnRH-I (de Koning *et al.*, 1978; Zilberstein *et al.*, 1983; King *et al.*, 1986, 1987; Guémené & Williams, 1992a). This possibility was assessed by challenging perifused pituitary tissue with three pulses of GnRH-I at intervals of 60-minutes.

Data is presented from a single experiment using pituitary tissues from adult and juvenile chickens of both sexes (FIGURE 4.4). Each pulse of GnRH-I was associated with a peak of LH release from the pituitary glands. Peaks of GnRH-I-induced LH secretion were larger in the adult cockerel (11 and 7-fold times basal LH for 10 and 1000 nM GnRH-I respectively) than in the laying hen (1.9 and 1.7-fold times basal LH for 10 and 1000 nM GnRH-I respectively). A concentration-dependency of GnRH-I on the magnitude of ΔLH was not seen in either sex. However the release of LH from pituitary tissue of the laying hen was typically slow during the declining portion of each GnRH-I-induced LH peak, and after stimulation with 1000 nM GnRH-I, the concentration of LH had not returned to a baseline when the next pulse of GnRH-I was introduced. The baseline release of LH from the GnRH-I-stimulated pituitary gland from laying hens but not from the adult cockerel tended to increase during the course of the perifusion.

There was a trend for the peak of the LH response of pituitary tissue from adult cockerels to decrease with each successive stimulation, but no effect of GnRH-I was seen on the responses of the pituitary gland of the laying hen.

The GnRH-I stimulated LH responses from pituitary glands of juvenile males and females were similar (FIGURE 4.4). The magnitude of LH release was the same and the LH peak size decreased with successive exposures to GnRH-I. This was more pronounced after stimulation with 1000 nM than with 10 nM GnRH-I.

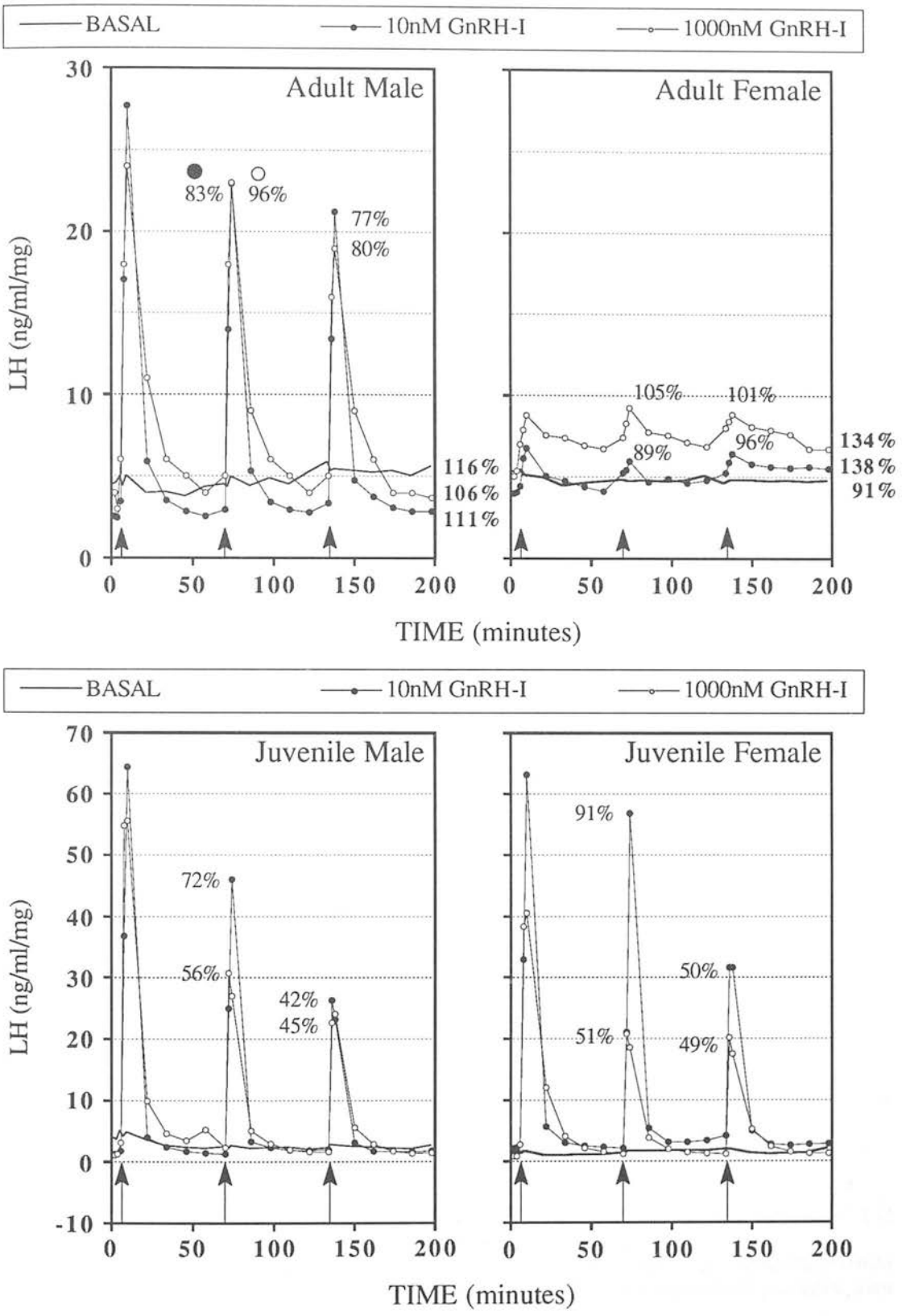


FIGURE 4.4: Effect of pulses of GnRH-I on LH release from pituitary glands of adult and juvenile cockerels and hens.
Quartered pituitary glands of adult or juvenile male and female chickens were perifused with three 4-min pulses (arrows) of GnRH-I at 60-min intervals. Two-min fractions were collected continuously or at intervals of 8-min. The amplitude of LH release is expressed as percent of the first LH peak (adjacent to each peak). The LH concentrations at the end of adult experiments are expressed as percent of starting concentration of LH (bold).

4.2.2.3 Sex differences in the depolarisation-induced release of LH from the adult pituitary gland

Pituitary tissues from adult chickens were perifused with veratridine or high K^+ to depolarise the cell membrane in order to establish whether the sexually differentiated profiles of GnRH-I-induced LH secretion are a function of the intracellular signalling mechanism by which GnRH-I stimulates LH release.

4.2.2.3.1 High K^+

Stimulation with 60 mM K^+ for 30-minutes produced a monophasic release of LH from pituitary glands from adult cockerels and laying hens (FIGURE 4.5), with more LH being released in the male (AUC = 601 ± 36) than the female (AUC = 135 ± 15; P<0.001).

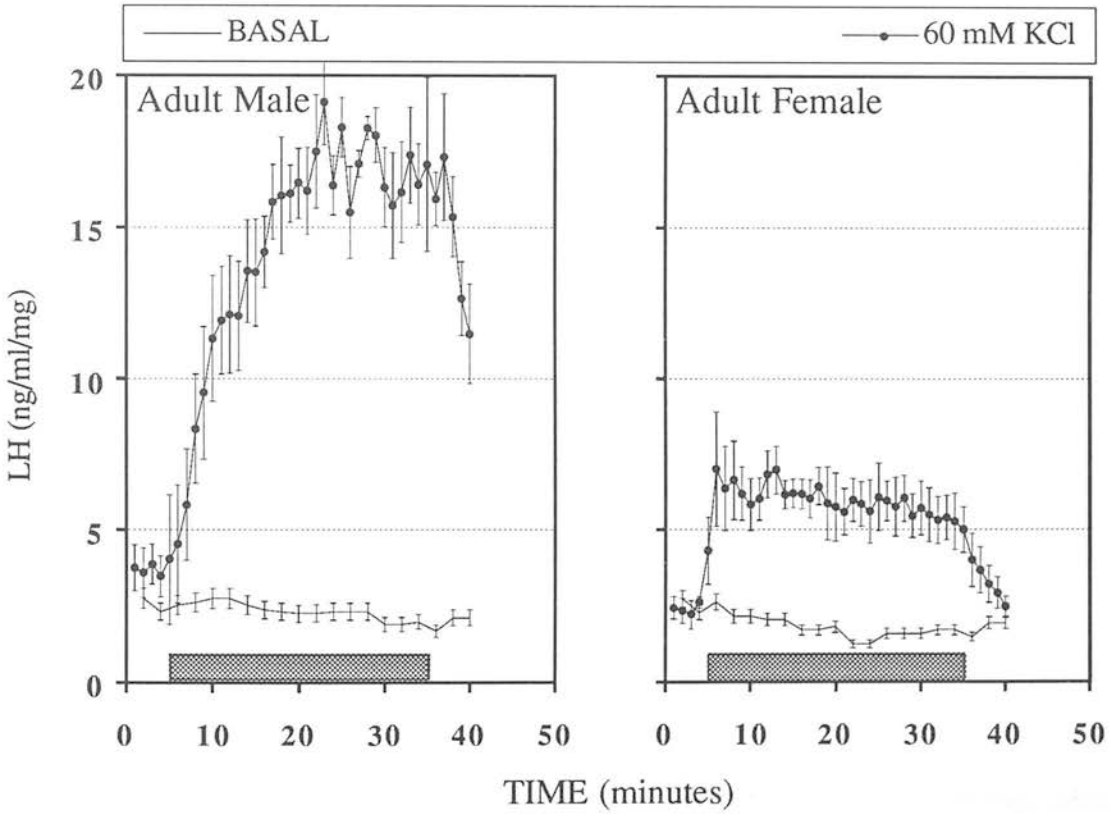


FIGURE 4.5: Effect of prolonged depolarisation with 60 mM K^+ on the release of LH from perifused pituitary glands of adult cockerels and hens.
300 µm pituitary slices from adult males and females were perifused with Avian Ringer containing 60 mM K^+ for 30-min (grey bar) before returning to standard Avian Ringer. One-min fractions of perifusate were collected (n = 3 per sex).

4.2.2.3.2 Veratridine

The depolarising agent veratridine stimulated a single phase of LH secretion from pituitary glands of adult males and females (FIGURE 4.6). The apparently larger release of LH from male pituitary

tissue than from female tissue, was not statistically significant ($\Delta AUC_{male} = 610 \pm 78$, $\Delta AUC_{female} = 382 \pm 39$; n = 3).

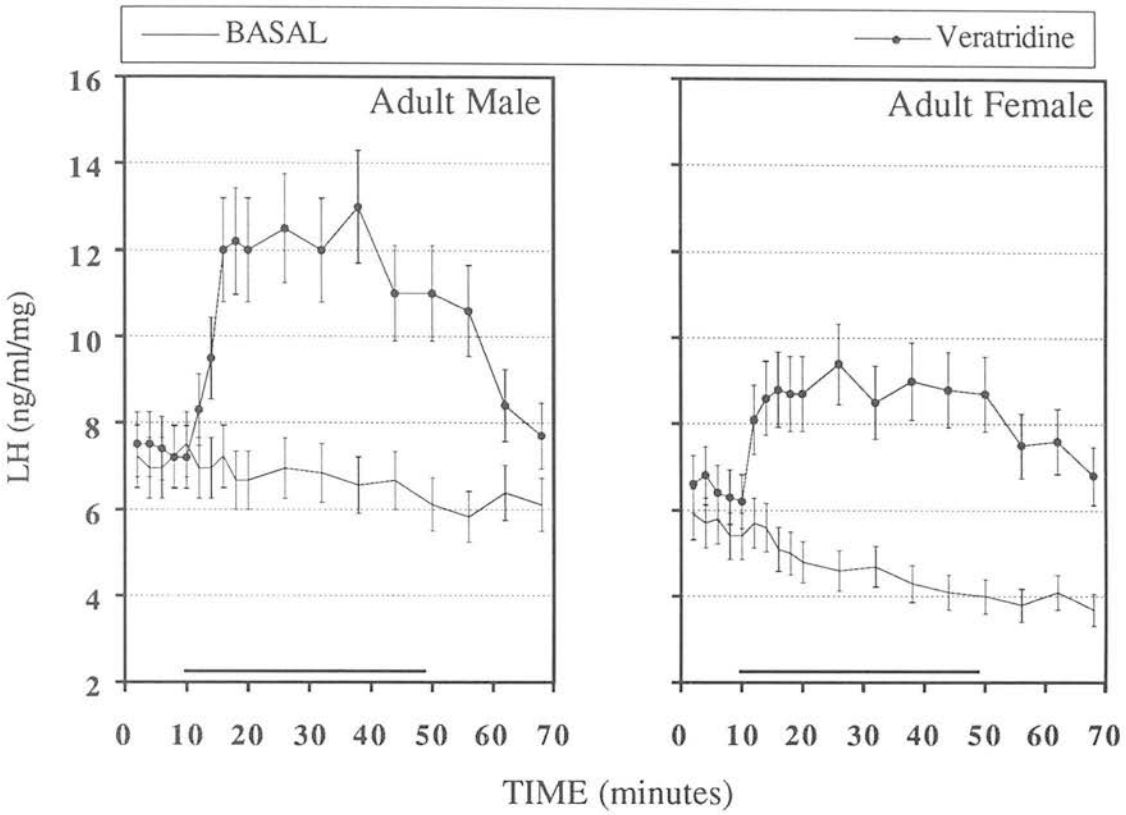


FIGURE 4.6: Veratridine-induced LH release from perifused pituitary glands from adult cockerels and hens.
Slices (300 µm transverse sections) of pituitary glands from adult males and females (n = 3 per treatment) were perifused with or without 10 µM veratridine for 40-min (grey bar). Two-min fractions of perifusate were collected continuously or at intervals of 4-min.

4.2.2.4 Sex difference in the ability of GnRH-I to maintain LH release from pituitary glands from adult chickens

The sex difference in the profile of GnRH-I-induced LH release from pituitary glands from adult chickens may be due to differences in the readily releaseable pools of LH. This possibility was investigated by determining the effect of long-term perifusion with GnRH-I on the readily releaseable pool of LH.

The plateau phase of LH released from pituitary glands from males perifused for 3-hours with GnRH-I declined slowly but remained constant for pituitary tissues from females (FIGURE 4.7). Withdrawal of GnRH-I immediately reduced the release of LH from pituitary tissue from chickens of both sex. Subsequent stimulation with a maximal dose of the depolarising agent veratridine (Davidson *et al.*, 1987a, 1988) increased LH secretion. In both sexes, the maximum concentrations of LH after exposure to veratridine were not significantly different from the LH

concentrations immediately prior to withdrawal of GnRH-I (Time = 190-minutes; FIGURE 4.7). However, the release of LH stimulated by veratridine from control male pituitary tissue was significantly more ($P<0.05$) than that released from male pituitary tissue stimulated previously with GnRH-I.

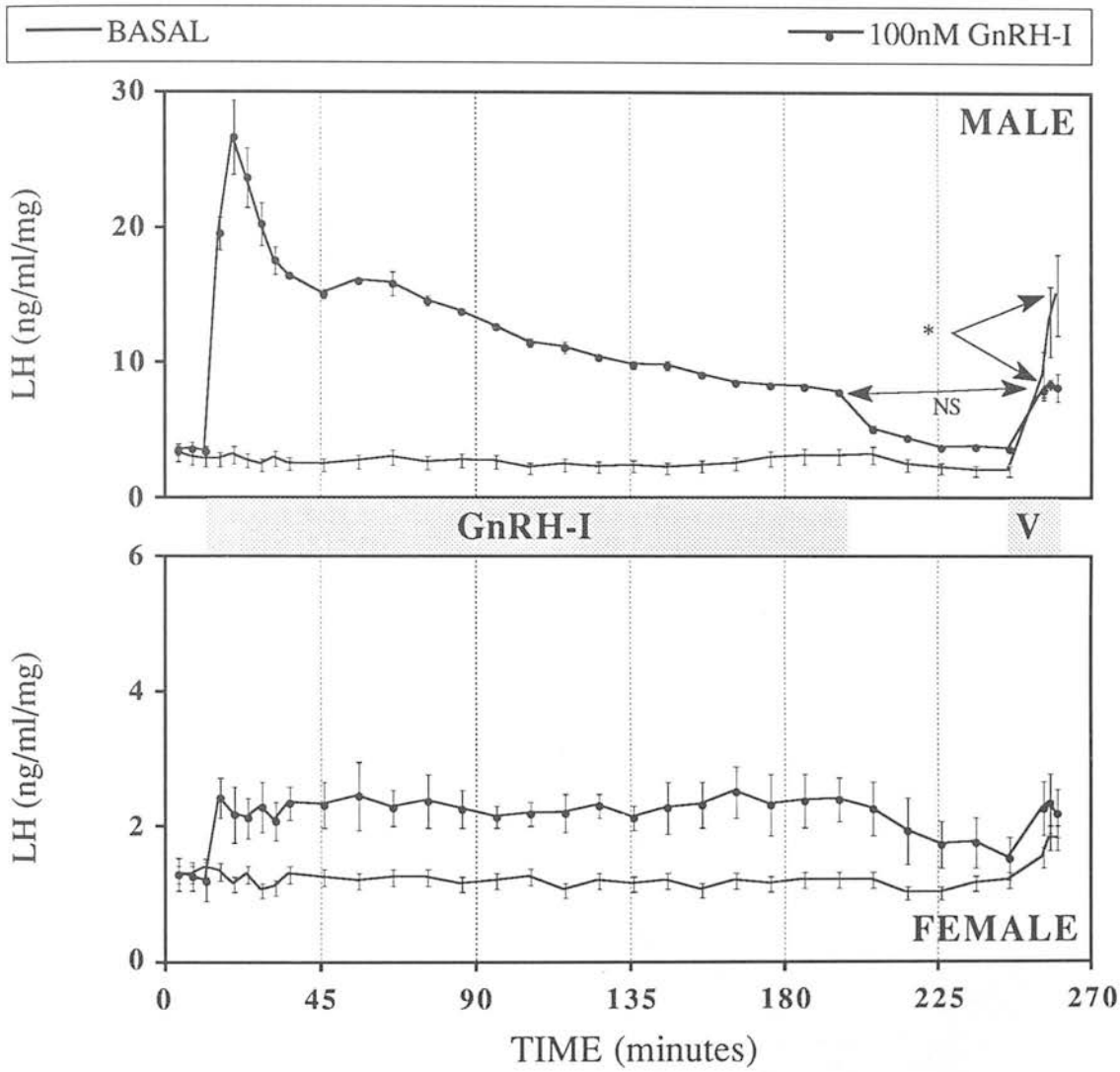


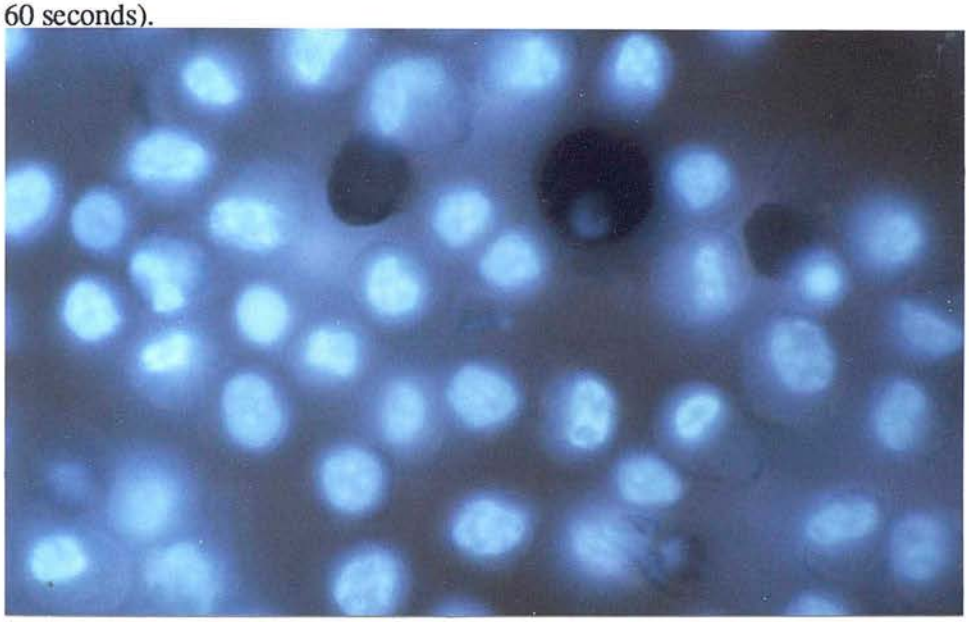
FIGURE 4.7: Sex difference in the profiles of LH release from pituitary glands of adult cockerels and laying hens stimulated by GnRH-I and veratridine.
Quartered pituitary glands from adult males and females were perifused with 100 nM GnRH-I for 3-h. One hour later, all pituitary glands were challenged with 30 μM veratridine (V). NS = not significantly different, *$P<0.05$ (n = 3). Note different scales on y-axes. Comparable results to these were seen in a similar experiment.

4.2.3 Comparison of the proportions of LH-containing cells in the pituitary glands of adult cockerels and laying hens

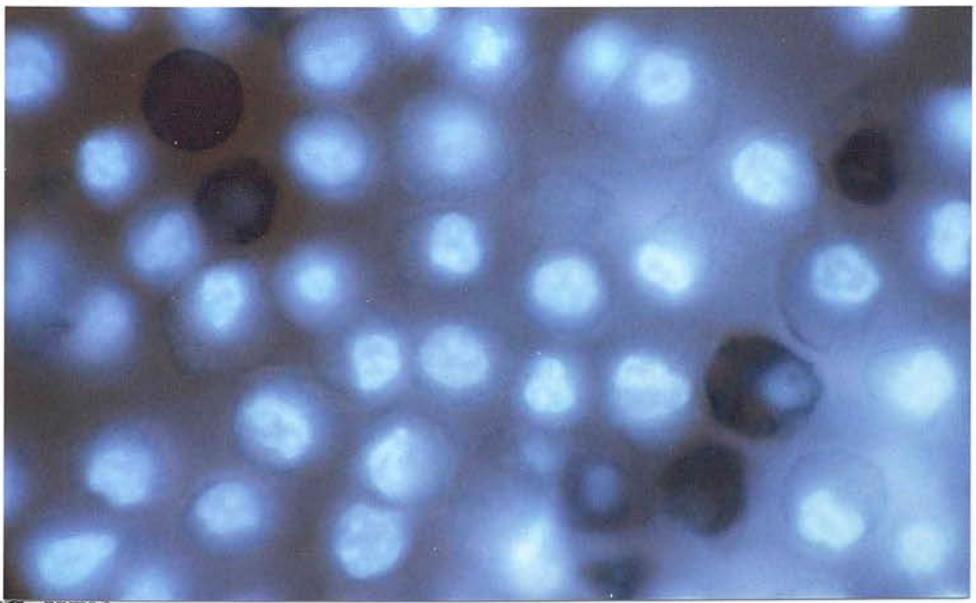
The lower content and readily releaseable pool of LH in the pituitary gland of the laying hen than in the adult cockerel may be due to a lower proportion of LH-containing cells. The proportion of LH-

containing cells was determined by immunocytochemistry using dispersed pituitary cells rather than sections of pituitary gland, to facilitate cell-counting. This trypsin-dissociation procedure does not affect the proportion of rat pituitary cell types by selective destruction of specific cells because the proportions correspond with those found in intact pituitary sections (Denef *et al.*, 1989).

The cytoplasm of LH-containing cells stained brown and contrasted against fluorescently stained nuclei (FIGURE 4.8). LH-containing cells represented 7.4 ± 1.0% and 7.8 ± 0.3% of the total pituitary cell number in respectively, the adult cockerel and laying hen. Gonadotroph cells of the adult hen required a higher concentration of LH-antiserum (1-in-5000) and more time to develop the peroxidase reaction complex (60 - 100 seconds), compared with cells of the male (1-in-10,000 and 30 - 60 seconds).



ADULT COCKEREL



LAYING HEN

FIGURE 4.8: LH-gonadotroph cells from dispersed pituitary glands of adult male and female chickens.
Pituitary glands from adult cockerels and laying hens were dispersed with trypsin and processed for LH-immunocytochemistry. LH-gonadotroph cells stain dark, and cell nuclei with a fluorescent marker. Magnification = x1099

4.2.4 Ultrastructural observations in gonadotroph cells from adult chickens

4.2.4.1 Sex differences in the ultrastructure of adult gonadotroph cells

The sex difference in the responsiveness of pituitary glands from adult chickens to GnRH-I may be due to a difference in the number or size of LH secretory granules in the gonadotroph cells. This possibility was investigated in a morphometric comparison of gonadotroph cells from laying hens and adult cockerels.

Morphometric methods have been applied to investigate the GnRH-I-induced changes in the numbers of secretory granules in the subplasmalemmal region of the cytoplasm in mouse gonadotroph cells (Lewis *et al.*, 1985, 1986), the maturational changes in granules of chicken somatotroph cells (Malamed *et al.*, 1985, 1988), the effect of GnRH stimulation on frog gonadotroph cells (Gracia-Navarro *et al.*, 1990), and the response of cultured ovine pituitary cells (Thorpe & Wallis, 1991). Stimulation of LH-gonadotroph cells with GnRH increases the number of secretory granules in a region of the cytoplasm close to the cell membrane, such that an even greater release of LH is induced by a subsequent challenge with GnRH (Lewis *et al.*, 1985, 1986). Gonadotroph cells therefore have a readily releaseable pool of LH which is related directly to pituitary responsiveness to GnRH (Lewis *et al.*, 1985, 1986). This view is supported by the observation that changes in pituitary responsiveness during the rat oestrous cycle are related to the number of LH secretory granules in the gonadotroph cells (Blake, 1980). The objective of the present study was to establish in gonadotroph cells of adult cockerels and hens, relationships between pituitary responsiveness and the distribution of secretory granules, and the content of pituitary LH with the number of granules.

The pituitary cell-types of the anterior pituitary gland were identified (FIGURES 4.9 and 4.10) according to the classification of Tai (1976), and Tai and Chadwick (1977) and included the Type-I (somatotrophs), Type-II (lactotrophs), Types-III and IV (respectively 'FSH' and 'LH' gonadotrophs) and Type-V (thyrotrophs). Only the Type-IV 'LH-cells' were subjected to morphometric analyses (TABLES 4.3 and 4.4).

TABLE 4.3: Sex difference in the size of LH-gonadotroph cells from adult cockerels and hens.

	Cell Area (μm^2)	Cell Diameter (μm)	Nuclear Area (μm^2)	Nuclear Diameter (μm)
MALE	71 ± 5	9.5 ± 0.2	16.0 ± 1.0	4.5 ± 0.2
FEMALE	59 ± 3*	8.7 ± 0.2*	20.3 ± 0.7*	5.1 ± 0.1*

Gonadotroph cells from adult chickens (7 - 9 cells per bird; 3 birds per sex) were measured by image analysis. *$P<0.05$ compared with the male.

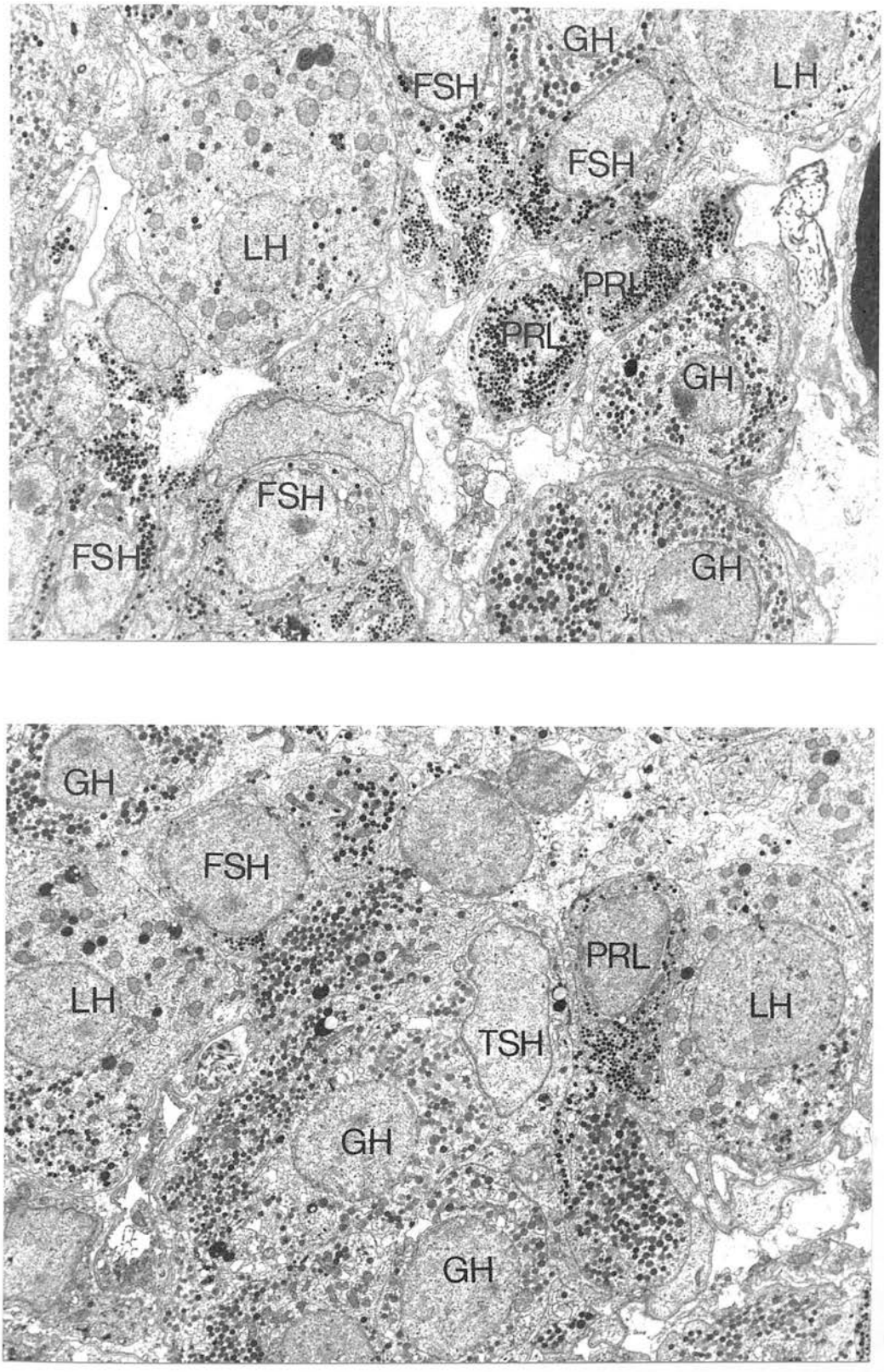


FIGURE 4.10: Ultrastructure of the anterior pituitary gland of the laying hen.
See FIGURE 4.9 for legend. Magnification = x4557

TABLE 4.4: Distribution and quantity of secretory granules in LH-gonadotroph cells from adult cockerels and hens.

		Granules/cell profile	Granule Density (per μm^2)	GFA (%)	ECD (nm)	Cytoplasmic Area (μm^2)
Total	Male	87 ± 13	1.6 ± 0.2	2.7 ± 0.3	143 ± 2	55 ± 4
	Female	$53 \pm 5^{*}$	1.4 ± 0.1^{NS}	2.9 ± 0.3^{NS}	$161 \pm 3^{***}$	$39 \pm 3^{**}$
Perinuclear	Male	20 ± 3	1.0 ± 0.1	1.9 ± 0.2	147 ± 3	19 ± 1
	Female	25 ± 2^{NS}	$1.6 \pm 0.2^{*}$	$3.6 \pm 0.3^{***}$	$163 \pm 4^{***}$	$15 \pm 1^{*}$
Subplasmalemma	Male	66 ± 12	1.8 ± 0.3	2.5 ± 0.4	141 ± 3	37 ± 3
	Female	$28 \pm 4^{***}$	1.2 ± 0.2^{NS}	2.4 ± 0.3^{NS}	$155 \pm 3^{**}$	$24 \pm 2^{**}$

Secretory granules were analysed in LH-gonadotroph cell profiles of pituitary glands from adult males and females. Granules were measured in the total, perinuclear and subplasmalemmal regions of the cytoplasm (excluding nucleus). NS = not significantly different, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the male (*t*-test, n = 22/26 cell profiles per sex; 7 - 9 cells per bird, 3 birds per sex). Granule density = density of granules in the cytoplasm. [number of granules/area cytoplasm]. GFA = Granule Fractional Area. Area of cytoplasm occupied by secretory granules. [100 x (number of granules x Mean Granule Area)/area cytoplasm]. Mean Granule Area = $[(ECD/2)^2 \times \pi]$. ECD = Equivalent Circular Diameter. Diameter derived from the circumference, by assuming a circular profile of granule.

'LH-gonadotrophs' were large rounded or elongated cells containing numerous organelles (FIGURES 4.11 and 4.12). Typically, there were 15 to 40 rounded or elongated mitochondria dispersed throughout the cytosol of the cell profile, and if the nucleus occupied a polar position, the mitochondria tended to reside in the area of the opposite pole. A further characteristic feature of these cells was the prominent smooth endoplasmic reticulum which was distended with moderately electron-dense granular material, and its presence was more pronounced in male LH-cells than those of the female. There was little sign of rough endoplasmic reticulum, although clusters of free ribosomes were present in the cytoplasm. Highly electron-dense secretory granules were distributed throughout the cytosol. Where the granules showed polarisation towards one end of the cell, they tended to lay close to an extracellular lumen (follicle) or a capillary vessel, and the nucleus occupied the other pole.

Gonadotroph cells from laying hens were significantly smaller ($P<0.05$), but had a larger nucleus ($P<0.05$) compared with gonadotroph cells from adult cockerels (TABLE 4.3). Larger secretory granules ($P<0.01$; TABLE 4.4) were observed in the perinuclear and subplasmalemmal regions of the cytoplasm of gonadotroph cells from laying hens than from adult cockerels. There were no differences in the diameters of secretory granules in the perinuclear or subplasmalemmal cytoplasm in LH-gonadotroph cells of either sex. Compared with the adult cockerel, the subplasmalemmal region of gonadotroph cells from laying hens contained fewer granules ($P<0.05$). The perinuclear cytoplasmic region of the LH-cells from females however, contained a higher density of secretory granules ($P<0.05$) which occupied a greater proportion of the cytoplasm (Granule Fractional Area; $P<0.001$) than in the male.

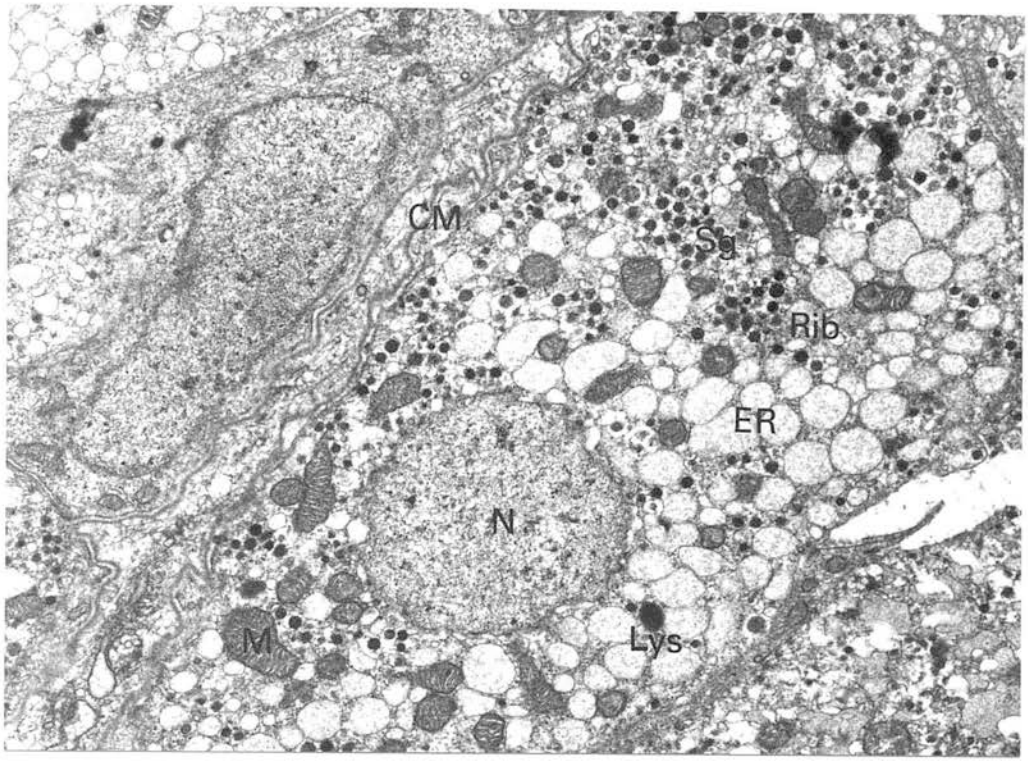


FIGURE 4.11: Ultrastructural features of LH-gonadotroph cells of adult cockerels.
Magnification = x9807. Cell membrane (CM), possible lysosome (Lys), mitochondrion (M), nucleus (N), ribosomes (Rib), smooth endoplasmic reticulum (SER), secretory granule (Sg).

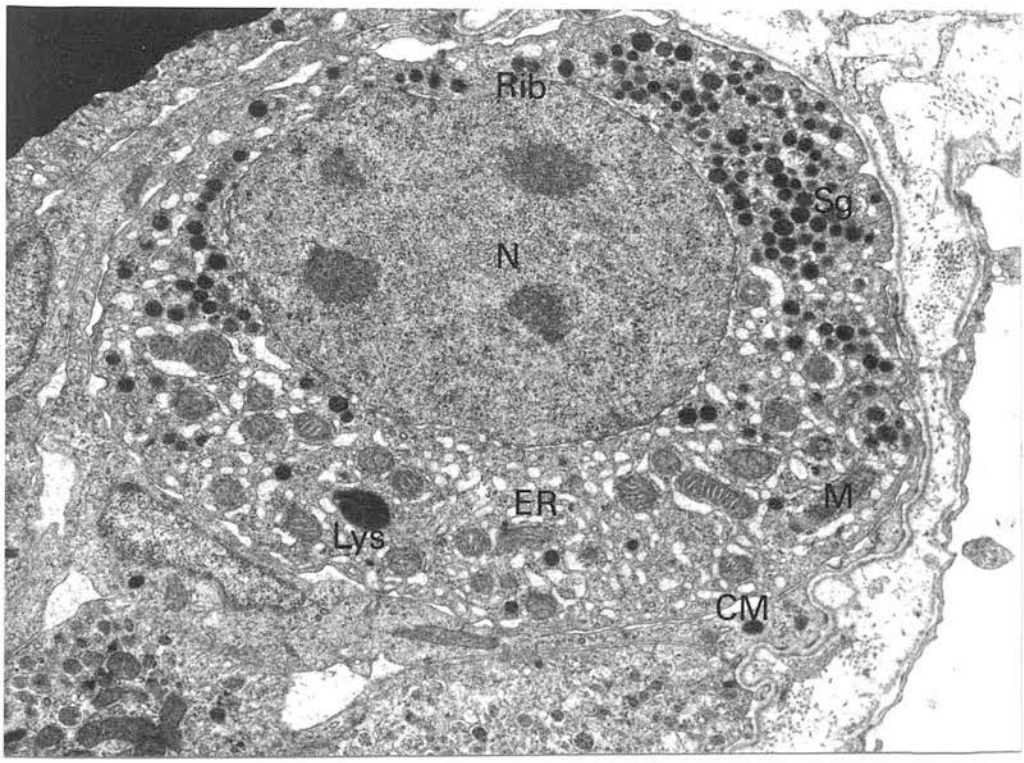


FIGURE 4.12: Ultrastructural features of LH-gonadotroph cells of laying hens.
Magnification = x10,882. See FIGURE 4.11 for legend.

4.2.4.2 Sex difference in lipid-containing cells in the anterior pituitary gland

An unusual pituitary cell-type found in the pituitary gland of adult cockerels (FIGURE 4.13) was not found in laying hens. A preliminary study indicated that these cells are not present in pituitary glands from juvenile (7-week old) male and female chickens. These LH-gonadotroph-like cells contained numerous small secretory granules (TABLE 4.5), but the most striking feature was the presence of large (1126 ± 77 nm in diameter; range 280 - 6326 nm; 1.65 ± 0.31 μm^2 in area; range 0.06 - 31.43 μm^2), heavily electron-dense structures in the endoplasmic reticulum. These bodies numbered 14.2 ± 2.6 per cell and ranged from being rounded to highly irregularly shaped material, sometimes appearing as multi-lobed structures (FIGURE 4.13). The inclusions were not bounded by an immediate membrane, but were enclosed within the membrane of the smooth endoplasmic reticulum (FIGURE 4.14). These inclusions were only occasionally associated with acid phosphatase-containing structures (FIGURE 4.15) but in general, there was no co-localisation of acid phosphatase with these inclusions (FIGURE 4.16). In cryostat sections of pituitary glands from adult cockerels, specific staining for lipid was demonstrated using osmium tetroxide (FIGURE 4.17).

TABLE 4.5: Comparison of the cell sizes and number of granules in LH-gonadotroph cells and lipid-containing cells in pituitary glands from adult cockerels.

	Cell Area	Nuclear Area	Cyto Area	Pn Area	Sp Area	Granules	ECD
LH-cell	71 ± 5	16 ± 1	55 ± 4	19 ± 1	37 ± 3	87 ± 13	143 ± 2
Lipid-cell	91 ± 8*	14 ± 2^{NS}	77 ± 8*	24 ± 3^{NS}	53 ± 5*	71 ± 15^{NS}	147 ± 4^{NS}

Pn = perinuclear, Sp = subplasmalemmal region of cytoplasm. Data for LH-gonadotroph cells of adult cockerels were taken from TABLES 4.3 and 4.4. Units given in TABLE 4.4. NS = not significantly different, *P<0.05; n = 25 lipid-containing cells.

4.2.4.3 Sex differences on the effects of GnRH-I on secretory granules in LH-gonadotroph cells in adult chickens

A study was carried out to establish whether the sexually differentiated biphasic or monophasic patterns of GnRH-I-induced LH secretion from the pituitary gland is related to changes in the distribution of LH secretory granules within the gonadotroph cells. Changes in the distribution of secretory granules were determined after stimulation with GnRH-I.

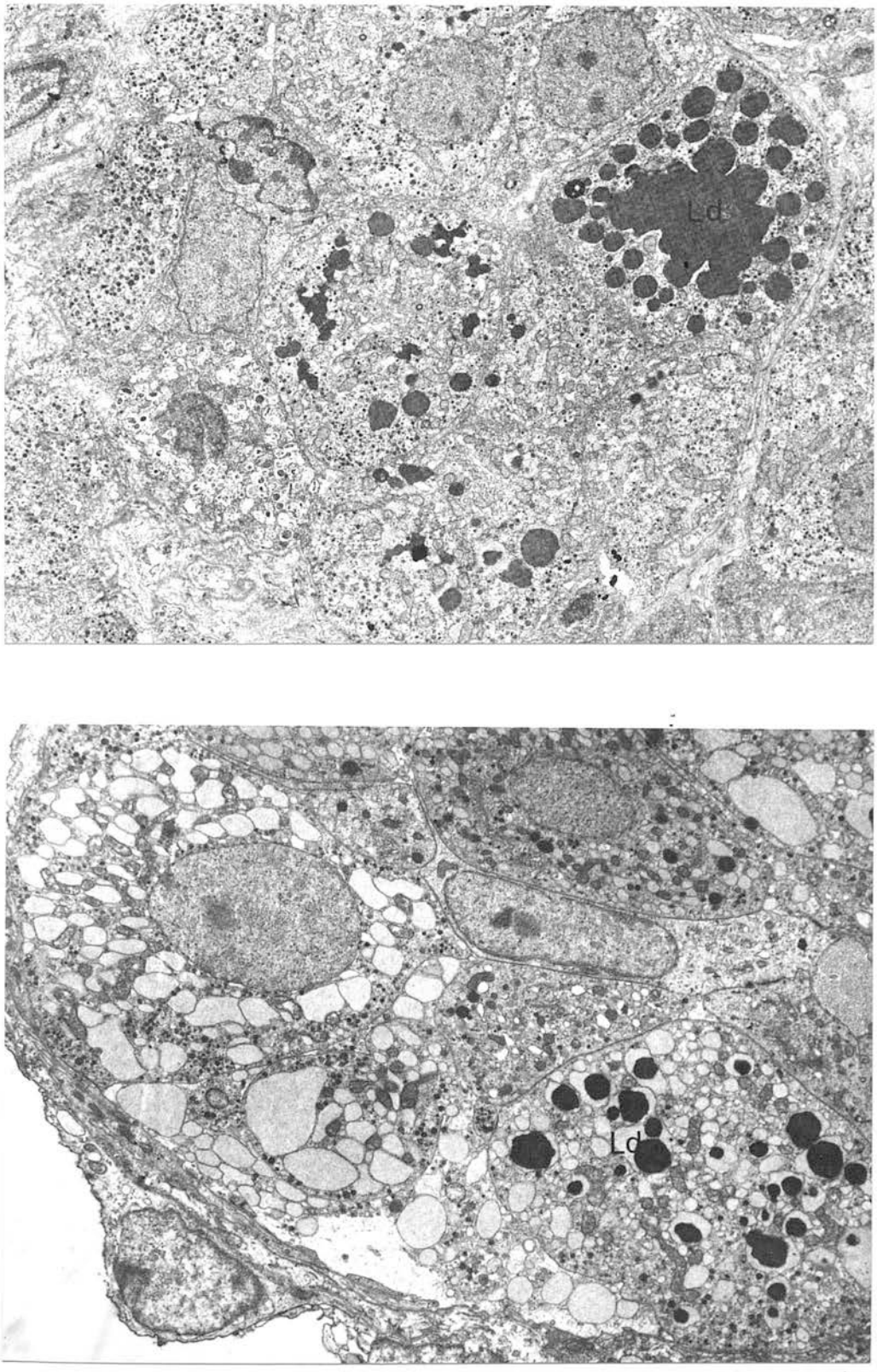


FIGURE 4.13: Putative lipid-containing cells of the adult cockerel pituitary gland.
Putative lipid (Ld; black areas). Magnification = (a) x4557 and (b) x4730.

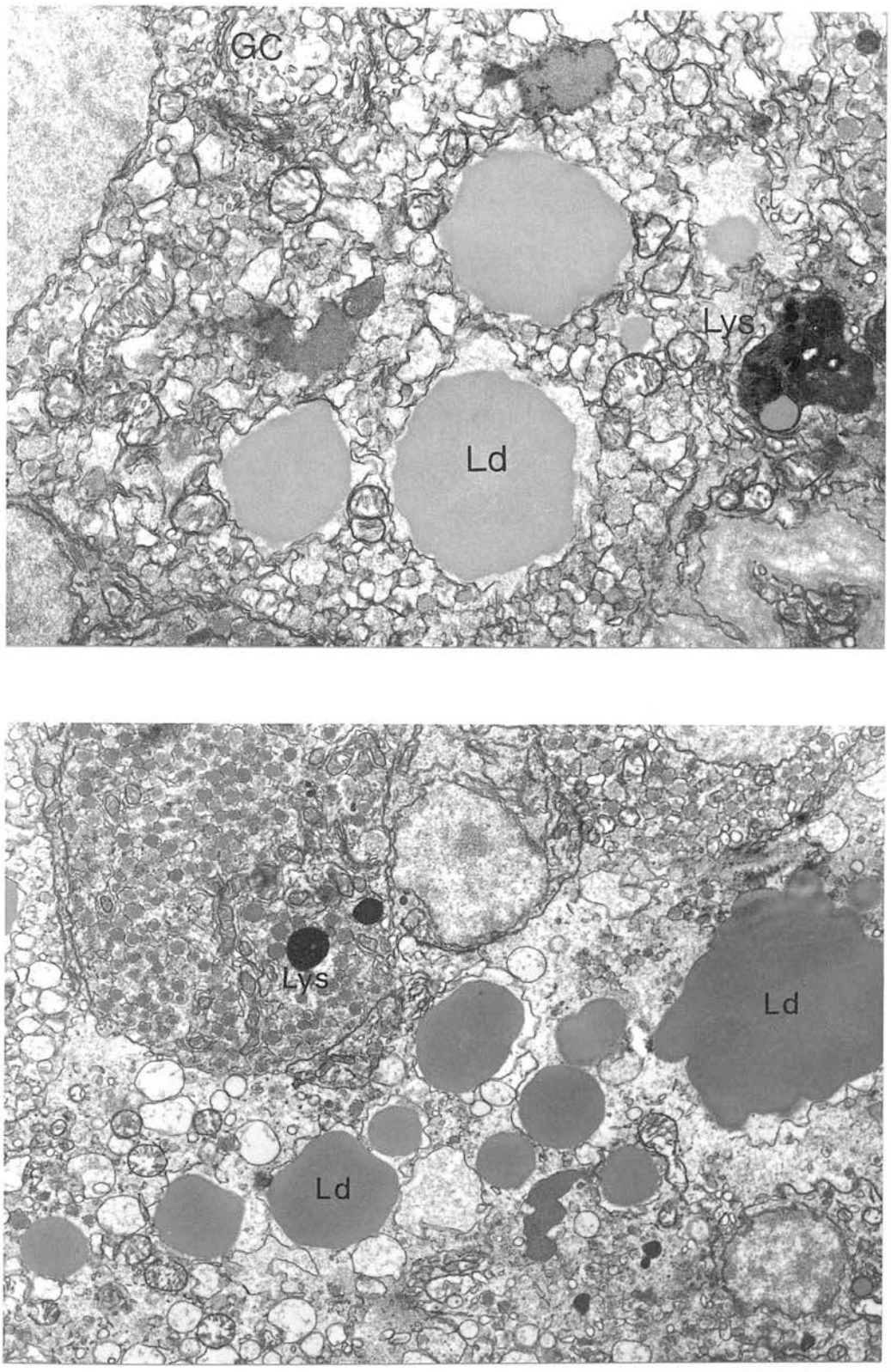


FIGURE 4.14: Membrane-bound nature of the putative lipid-containing bodies of the adult cockerel pituitary gland.
Specific membrane staining contrasts against low electron-density lipid material (Ld). Possible lysosome (Lys). (a) magnification = x7473, (b) magnification = x15,898.

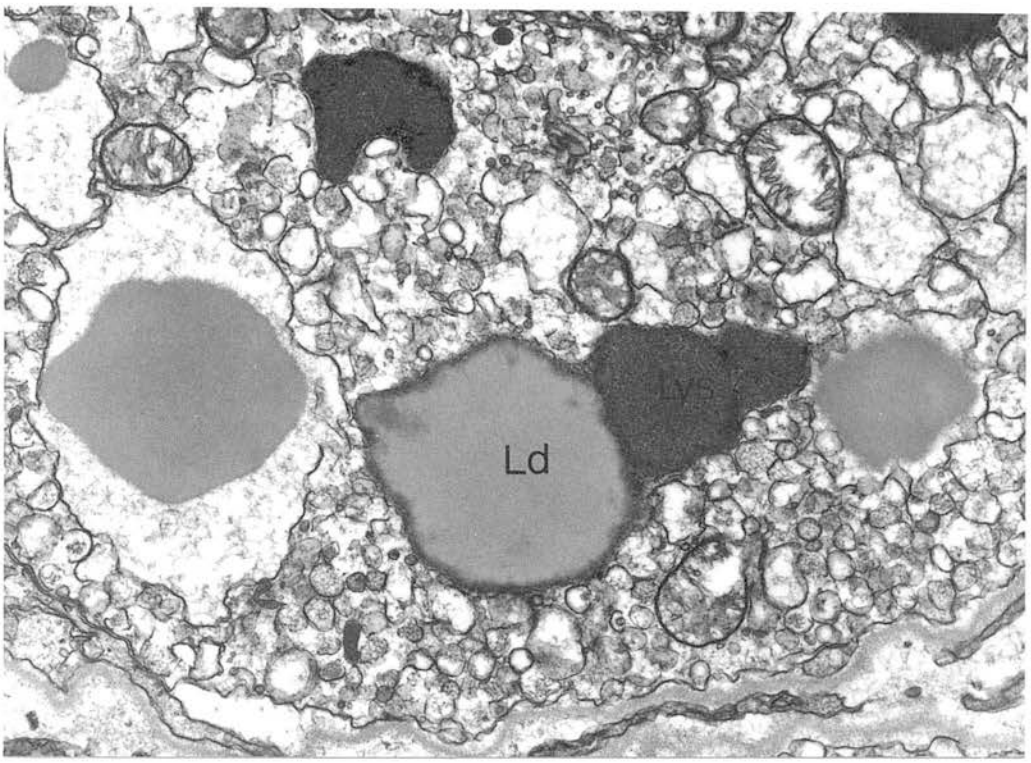


FIGURE 4.15: Possible lysosomal association of the putative lipid-containing bodies of the adult cockerel pituitary gland.
See FIGURE 4.14 for legend. Magnification = x18,582.

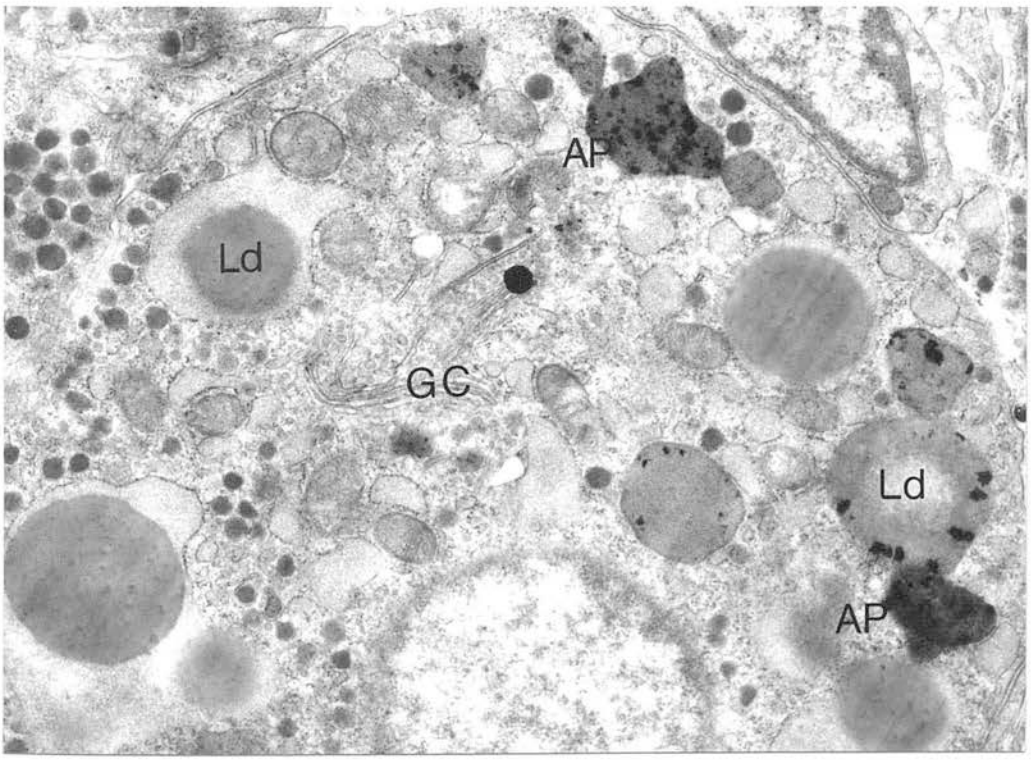


FIGURE 4.16: Occasional co-localisation of acid phosphatase-immunoreactivity with putative lipid-containing bodies of the adult cockerel pituitary gland.
Identification of acid phosphatase (AC) -containing lysosomes in lipid (Ld) - containing pituitary cells. Golgi complex (GC). Magnification = x19,484.

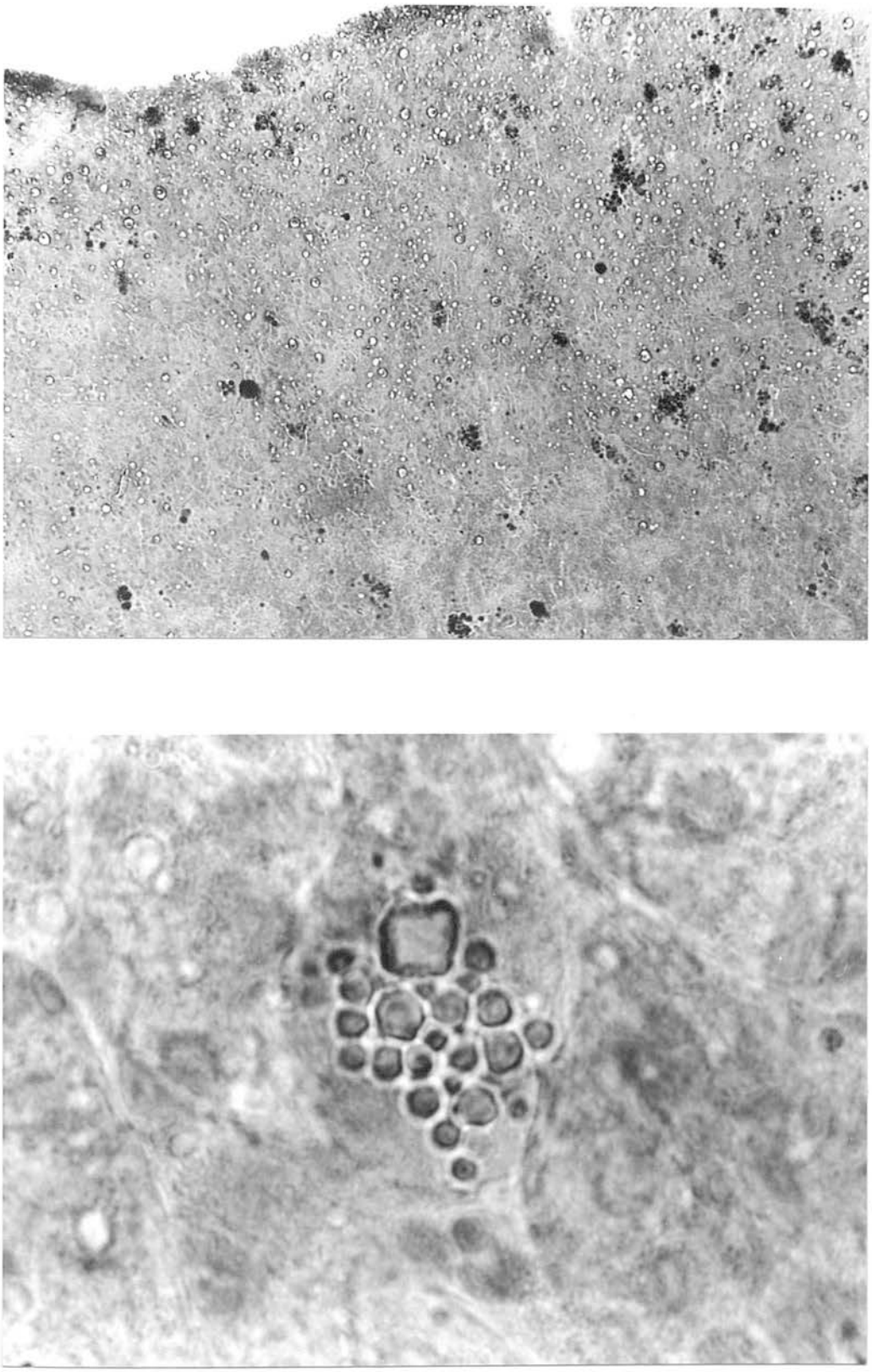


FIGURE 4.17: Osmiophilic property of lipid-containing bodies of the adult cockerel pituitary gland.
Light microscopy of osmium tetroxide-stained cryostat sections of anterior pituitary gland. Black areas of staining indicate lipid. (a) magnification = x191, (b) magnification = x478.

GnRH-I (20 µg/kg) increased the plasma concentration of LH within 2-minutes of injection in both sexes ($P<0.05$; FIGURE 4.18), however at 30-minutes, whereas the concentration was returning to baseline in the male, the concentration of LH remained high in the female.

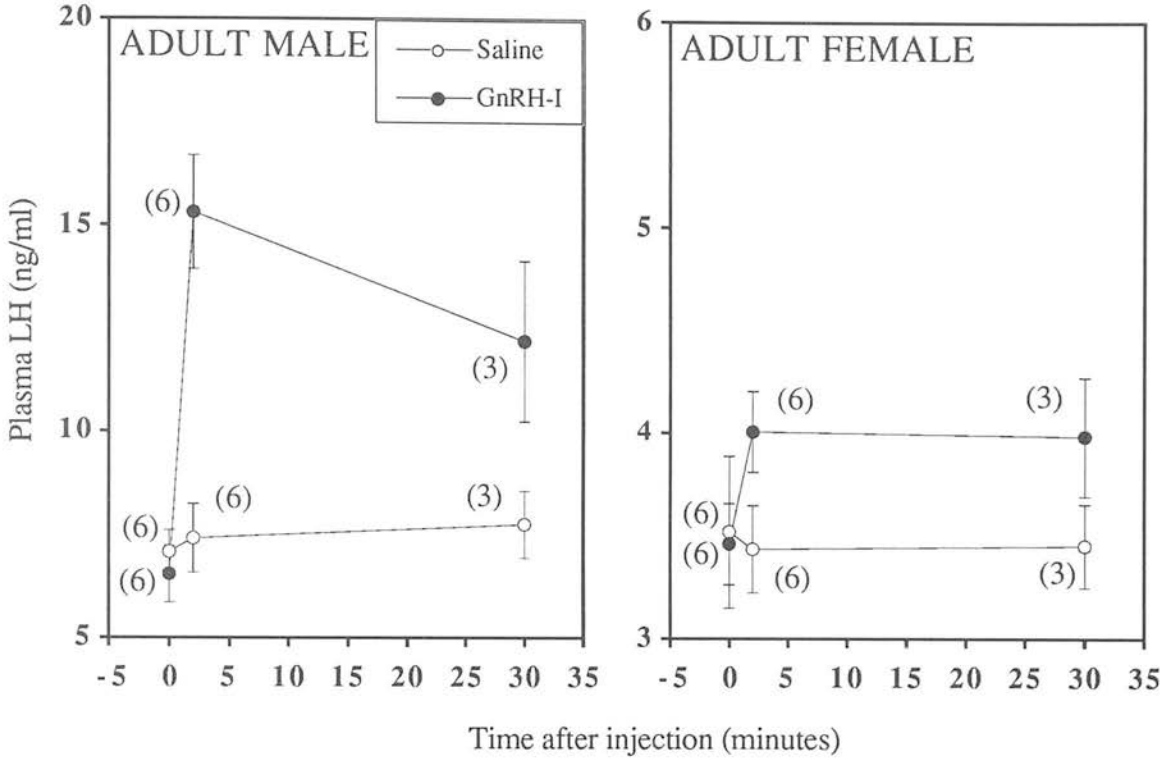


FIGURE 4.18: GnRH-I-induced LH secretion from pituitary glands of adult cockerels and hens taken for ultrastructural analysis of secretory granules in gonadotroph cells. 20 µg GnRH-I/kg body weight or saline was injected intravenously into adult cockerels and hens at time zero. Birds were killed at 2 or 30-mins after injection. Note difference in scale on y-axes. The number of measurements per sex per treatment is given on the graphs in parentheses. Pituitary glands were processed for transmission electron microscopy and morphometric measurements (see below).

The size of gonadotroph cells from adult cockerels and laying hens increased within 2-minutes of injection of GnRH-I, but this was only statistically significant in the hen ($P<0.05$; TABLE 4.6). No significant changes were found in the cockerel however trends could be identified. The number of secretory granules in the subplasmalemmel region of the cytoplasm in gonadotroph cells of both sexes increased at 2 and 30-minutes after injection of GnRH-I (TABLES 4.7 and 4.8). Two-minutes after injection of GnRH-I, there was a transient reduction in the density of secretory granules in the perinuclear region of gonadotroph cells of the laying hen ($P<0.05$). With this exception, GnRH-I did not generally affect the granule density of the gonadotroph cells of cockerels or hens. GnRH-I did not affect the size of the granules, or the fractional area of cytoplasm occupied by the granules in the perinuclear or subplasmalemmal regions, and did not affect the size of the cell nucleus.

TABLE 4.6: Comparison of the size of gonadotroph cells from adult cockerels and laying hens after GnRH-I injection.

Time after injection		Cell Area (μm^2)	Nuclear Area (μm^2)	Cell Diameter (μm)	Nuclear Diameter (μm)
MALE	Control	71 ± 4	16 ± 1	9.5 ± 0.2	4.5 ± 0.2
	2-min	87 ± 7^{NS}	19 ± 1	10.6 ± 0.5^{NS}	4.9 ± 0.3
	30-min	85 ± 8^{NS}	17 ± 1	10.4 ± 0.6^{NS}	4.7 ± 0.3
FEMALE	Control	59 ± 3	20 ± 1	8.7 ± 0.2	5.0 ± 0.1
	2-min	$82 \pm 5^{*}$	22 ± 1	$10.2 \pm 0.3^{*}$	5.3 ± 0.1
	30-min	$78 \pm 6^{*}$	19 ± 2	$10.0 \pm 0.4^{*}$	4.9 ± 0.3

Measurements were taken of whole cell profiles which included a nuclear profile (n = 7 - 9 cells per treatment per sex). NS = not significantly different, *P<0.05, **P<0.01 compared with sex-matched control.

TABLE 4.7: Effect of GnRH-I injection on the ultrastructural morphology of gonadotroph cells from laying hens.

Time after injection		Gran. No.	Density	GFA (%)	Cyto Area (μm^2)
Total	Control	53 ± 5	1.4 ± 0.2	2.9 ± 0.3	39 ± 3
	2-min	73 ± 11^{NS}	1.2 ± 0.2^{NS}	2.8 ± 0.4	$59 \pm 4^{**}$
	30-min	72 ± 8^{NS}	1.4 ± 0.2^{NS}	2.9 ± 0.5	$60 \pm 6^{*}$
Perinuclear	Control	25 ± 2	1.7 ± 0.2	3.6 ± 0.3	15 ± 1
	2-min	26 ± 4^{NS}	$1.2 \pm 0.2^{*}$	$2.6 \pm 0.3^{*}$	$22 \pm 1^{**}$
	30-min	25 ± 4^{NS}	1.4 ± 0.3^{NS}	2.8 ± 0.5^{NS}	$21 \pm 2^{*}$
Subplasmalemma	Control	28 ± 4	1.2 ± 0.2	2.4 ± 0.3	24 ± 2
	2-min	$48 \pm 7^{*}$	1.3 ± 0.2^{NS}	2.8 ± 0.4	$37 \pm 3^{**}$
	30-min	$47 \pm 5^{**}$	1.4 ± 0.2^{NS}	2.9 ± 0.5	$39 \pm 5^{*}$

GFA = Granule Fractional Area. NS = not significantly different, *P<0.05, **P<0.01 compared with control. Measurements were taken of whole cell profiles which included a nuclear profile (n = 22 - 28 cell profiles per treatment).

TABLE 4.8: Effect of GnRH-I injection on the ultrastructural morphology of gonadotroph cells from adult cockerels.

Time after injection		Gran. No.	Density	GFA (%)	Cyto Area (μm^2)
Total	Control	87 ± 13	1.6 ± 0.2	2.7 ± 0.3	55 ± 4
	2-min	109 ± 18^{NS}	1.4 ± 0.3^{NS}	2.7 ± 0.3	67 ± 6^{NS}
	30-min	107 ± 11^{NS}	1.6 ± 0.2^{NS}	2.8 ± 0.3	69 ± 7^{NS}
Perinuclear	Control	20 ± 3	1.0 ± 0.2	1.9 ± 0.2	19 ± 1
	2-min	21 ± 5^{NS}	1.0 ± 0.1^{NS}	1.7 ± 0.3	22 ± 2^{NS}
	30-min	28 ± 4^{NS}	1.4 ± 0.3^{NS}	2.4 ± 0.4	23 ± 2^{NS}
Subplasmalemma	Control	66 ± 12	1.8 ± 0.3	3.0 ± 0.4	37 ± 3
	2-min	88 ± 16^{NS}	1.7 ± 0.4^{NS}	3.2 ± 0.4	45 ± 4^{NS}
	30-min	79 ± 10^{NS}	1.8 ± 0.2^{NS}	2.9 ± 0.4	46 ± 3^{NS}

See TABLE 4.7 for legend. (n = 20 - 24 cells per treatment).

4.2.5 Studies on pituitary GnRH receptors

The sex differences in the responses of pituitary glands from adult chickens to GnRH-I could be due to differences in the binding sites for GnRH-I in the pituitary gland. The GnRH-receptors of the chicken pituitary gland have not been characterised due to the lack of a sufficiently sensitive receptor assay. A series of experiments were initiated to characterise GnRH-receptors in the chicken pituitary gland.

In preliminary experiments using four different iodinated GnRH analogues, only Buserelin and [D-Trp^6]-GnRH-ethylamide (D-Trp^6-Etamide) showed specific binding above a non-specific binding of 3000 - 5000 cpm (FIGURE 4.19). However, the specific binding did not increase linearly with increasing volume of pituitary homogenate. These radio-iodinated analogues showed satisfactory binding to human placental membranes and rat pituitary membranes (personal communication, TA Bramley). Little or no specific binding to chicken pituitary homogenate was found using chicken GnRH-II and mGnRH tracers.

Binding of ^{125}I-GnRH-II or ^{125}I-Buserelin to chicken pituitary homogenate was not increased by peptidase inhibitors such as EDTA, phenylmethyl sulphonic fluoride, N-ethyl-maleide, pepstatin or N-tosyl-L-phenylalanine chloromethyl ketone (0.5 mM).

D-Arg^6-GnRH-II is more resistant to metabolic degradation than is GnRH-II (Millar *et al.*, 1986; Sharp *et al.*, 1986b). The conditions for radiolabelling and HPLC purification of the ligand were

established. However, neither this fraction or the five other peaks of radioactivity collected, bound to homogenates of juvenile chicken pituitary gland.

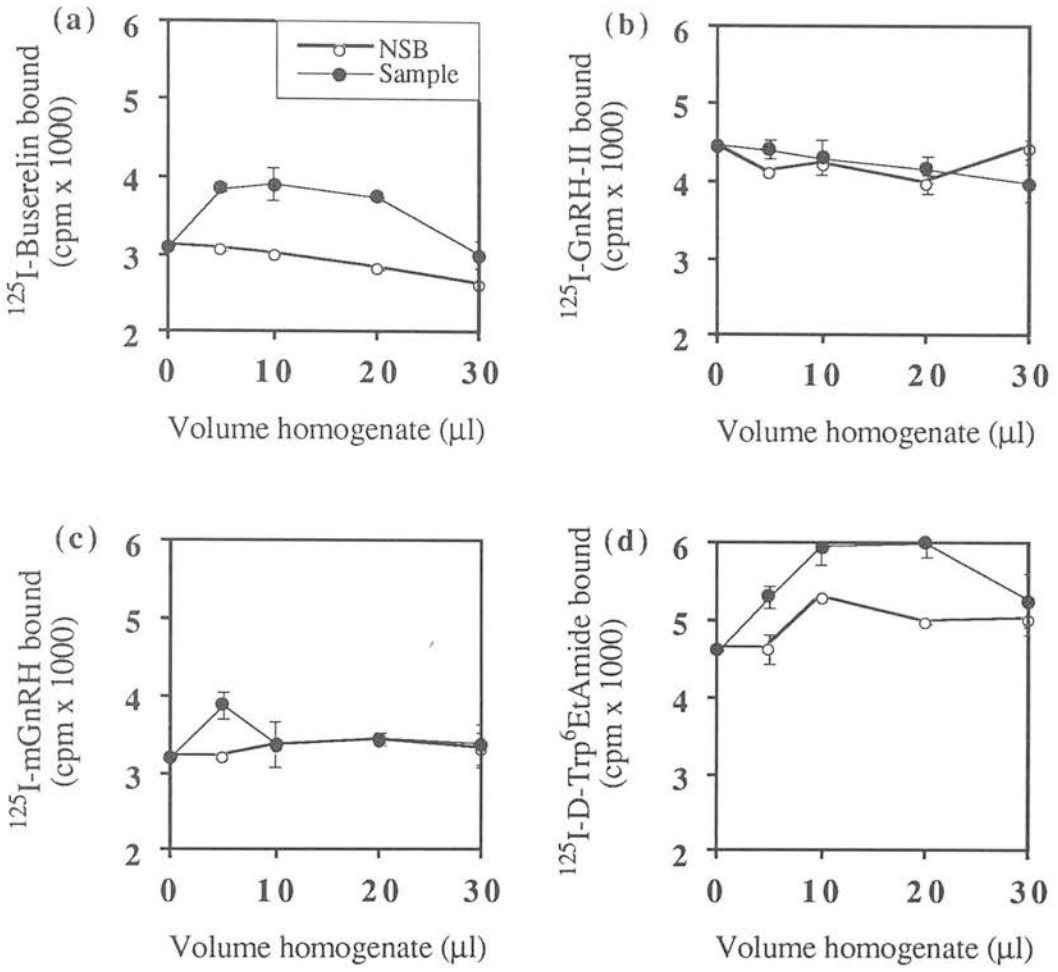


FIGURE 4.19: Binding characteristics of four GnRH tracers to chicken pituitary tissue.
Homogenates of pituitary glands from juvenile chickens were incubated with radiolabelled (a) Buserelin, (b) GnRH-II, (c) mGnRH or (d) D-Trp^{6}-EtAmide in the presence (NSB) and absence (sample) of non-labelled ligand. The mean activity bound is shown in counts per minute (cpm; n = 3).

Scatchard analysis showed little evidence for specific GnRH receptors in the chicken pituitary gland using ^{125}I-labelled [D-Trp^{6}]-GnRH-ethylamide (FIGURE 4.20). However up to 6000 cpm of the binding to pituitary homogenate could be displaced by unlabelled peptide. The technique was validated by including rat pituitary homogenate in a parallel set of tubes, for which K_a was calculated (normal range = 0.1 - 0.3 nM; Marian *et al.*, 1981).

The failure to establish an assay for GnRH-binding sites in anterior pituitary glands made it impossible to determine whether GnRH-binding sites are sexually differentiated in adult chickens.

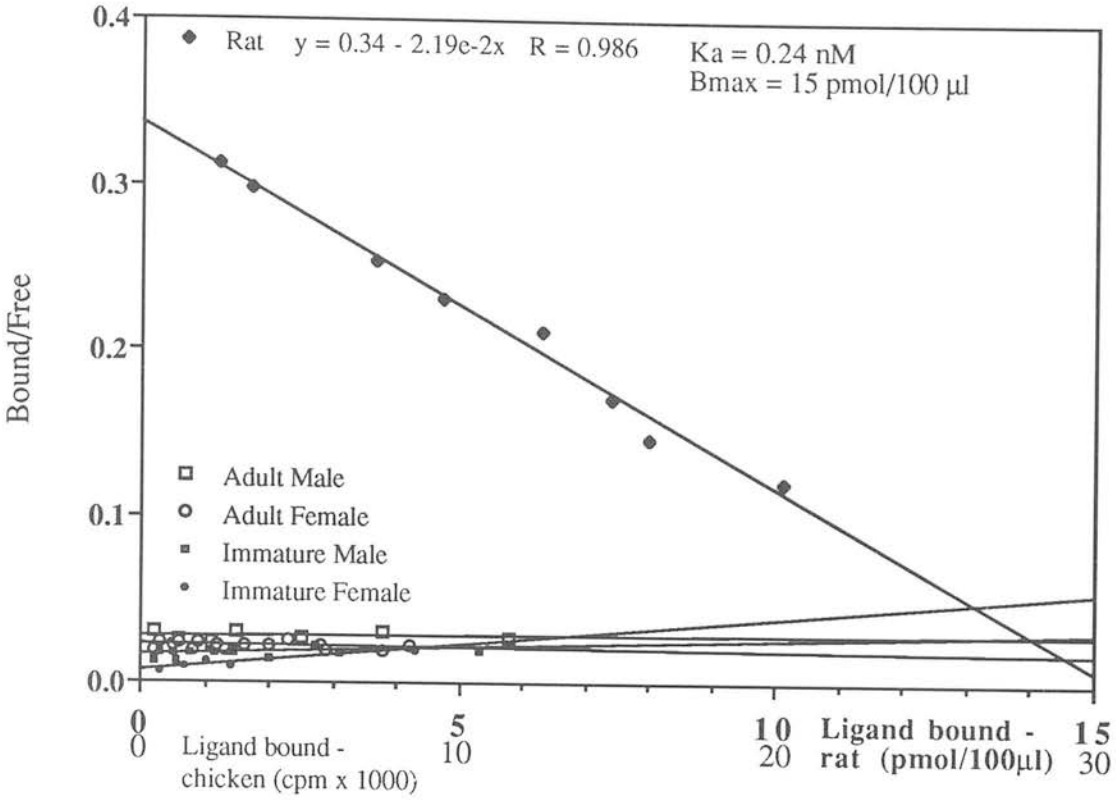


FIGURE 4.20: Scatchard plots of ^{125}I-labelled [D-Trp^6]-GnRH-ethylamide binding in homogenates of pituitary glands from chickens and rats.
Samples of pituitary homogenate were incubated with 1 ml buffer containing 10,000 - 200,000 cpm ^{125}I-labelled [D-Trp^6]-GnRH-ethylamide at 4°C for 2-h ± 10 µg unlabelled peptide. Scatchard analyses were performed on the specifically bound activity of labelled analogue to pituitary homogenate. Correlation coefficients for lines fitted to the chicken pituitary data ranged between R = 0.016 - 0.908.

4.3 DISCUSSION

4.3.1 Sex differences in the baseline and GnRH-I-stimulated magnitude of LH release from the pituitary gland *in vitro*

The sexually differentiated baseline concentration of LH and the GnRH-I-induced LH responses of the adult chicken *in vivo* (CHAPTER 3) were partly a function of the anterior pituitary gland. In CHAPTER 3, the regulation of baseline concentrations of plasma LH was shown to be at least partly independent of GnRH-I in adult chickens, and particularly in laying hens. The present results *in vitro* indicate that the basal release of LH from unstimulated pituitary glands from adult and juvenile chickens of both sexes, is related to the baseline concentration of plasma LH. It is likely that this GnRH-I-independent release of LH from the pituitary gland contributes to the baseline concentration of plasma LH.

In CHAPTER 3, it was suggested that the sexually differentiated responsiveness of the pituitary gland to GnRH-I is related to the sexually differentiated concentration of pituitary LH. However,

not all the LH is available for immediate release because LH may not be packaged in a suitable form. This could be because post-translational processing (e.g. glycosylation or sialylation) of the hormone is incomplete, or because the hormone is positioned away from the plasma membrane in a 'storage' pool (Bremner & Paulsen, 1974; Hoff *et al.*, 1977; Liu & Jackson, 1978; Adams & Nett, 1979; Pickering & Fink, 1979b; Lewis *et al.*, 1985, 1986; Gracia-Navarro *et al.*, 1990). The isolated pituitary gland was therefore depolarised with K^+ to estimate the readily releaseable pool (RRP) of LH. The pituitary gland of the laying hen contains about 9-times less RRP of LH than that of the adult cockerel.

The sex difference in content of pituitary LH in adult chickens was not due to a difference in the proportion of LH-containing gonadotroph cells in the pituitary gland. This suggests that the lower concentration of pituitary LH in laying hens compared with adult cockerels is due to a lower LH content in each gonadotroph cell. The morphometric data support this deduction and also show that there are fewer secretory granules in the subplasmalemmal region of LH-gonadotroph cells of laying hens than in those of adult cockerels. This subplasmalemmal region of the cytoplasm corresponds to the readily releaseable pool of LH in mouse gonadotroph cells (Lewis *et al.*, 1985, 1986). Also, in the immunocytochemistry study, a higher concentration and a longer incubation time with anti-LH was required for the development of a peroxidase reaction product for pituitary cells of the laying hen than those of the adult cockerel. These observations indicate that there is less LH-immunoreactivity in the pituitary cells of laying hens than of adult cockerels.

It is concluded that GnRH-I stimulates a smaller release of LH in the laying hen *in vivo* and *in vitro* simply because there is less pituitary LH available for immediate release compared with that in the adult cockerel. Likewise, the similar amounts of total and RRP of pituitary LH in juvenile male and female chickens explains the similar amounts of LH released following stimulation with GnRH-I *in vivo* and *in vitro*.

However, a discrepancy was described (SECTION 3.3.2) between the sexually differentiated maximum incremental change in plasma LH stimulated by GnRH-I in juvenile chickens, and the absence of a sex difference in the ΔAUC of LH. It was suggested that this discrepancy might be off-set by a more prolonged time-course of LH secretion from the pituitary gland of juvenile hens, and supported by the slower rate of decline in the falling phase of plasma LH in juvenile hens than in juvenile cockerels (SECTION 3.3.2). However, GnRH-I stimulated similar profiles of LH release *in vitro* in both of the juvenile sexes and therefore does not support this suggestion. This means that the sex difference in the pattern of LH secretion in the juvenile chicken *in vivo* may be due to a difference in the rate of LH clearance from plasma rather than a difference in the secretion of LH from the pituitary gland.

4.3.2 Sex differences in pituitary sensitivity to GnRH-I

A further feature of the sex difference, which was confirmed *in vitro*, is the lower sensitivity of laying hens to GnRH-I compared with adult cockerels (Sharp *et al.*, 1987). The sensitivity of gonadotroph cells to GnRH may relate to the number of GnRH receptors or the affinity for its ligand (Zilberstein *et al.*, 1983) or depend on the efficacy of post-receptor pathways (Smith *et al.*, 1983; Gorospe & Conn, 1987a, 1987b, 1988; Chang *et al.*, 1988a). Unfortunately, in agreement with previous work (Millar & King, 1983; Millar *et al.*, 1986; King *et al.*, 1989), a study of GnRH-receptors in the chicken pituitary gland was not possible due to the low affinity of the receptors for several GnRH ligands. However, a method has been described recently which claims success in characterising these pituitary receptors in different physiological states and after steroid treatment (Kawashima *et al.*, 1992a).

A number of criticisms can be made of these claims which are inconsistent with both classical receptor theory and the sensitivity limitations normally associated with current GnRH radio-receptor assays. Firstly, by plotting all the quoted K_a values against their respective B_{max} values (Kawashima *et al.*, 1992a), a straight line is obtained with a correlation coefficient of 0.925 (FIGURE 4.21). This demonstrates that K_a and B_{max} are *directly related*, which is inconsistent with what is known of receptor kinetics (Hulme & Birdsall, 1992) and suggests that GnRH-receptors were probably not being measured. The second point concerns the high degree of accuracy reflected in the very small experimental errors; a feature atypical of Scatchard analyses (TA Bramley, personal communication). Furthermore, these authors quote a bound/free ratio of 0.006 (an indication of the maximum amount of hormone bound; Scatchard, 1949) which again is a reflection on the accuracy of their measurements (compared with a ratio of 0.34 of specific binding to GnRH of rat pituitary tissue; FIGURE 4.20). Thus according to Kawashima *et al.* (1992a), out of 100,000 cpm of GnRH label added to chicken pituitary membranes, only a maximum of 600 cpm would be *specifically* bound above a background activity of 99,400 cpm. Moreover, in view of the quoted counting efficiency of 65 - 76% (Kawashima *et al.*, 1992a), this makes for an inaccurate and highly unreliable assay technique. It is therefore difficult to discuss these observations with any confidence. It is concluded that GnRH-receptors remain to be fully characterised in the pituitary gland of the chicken.

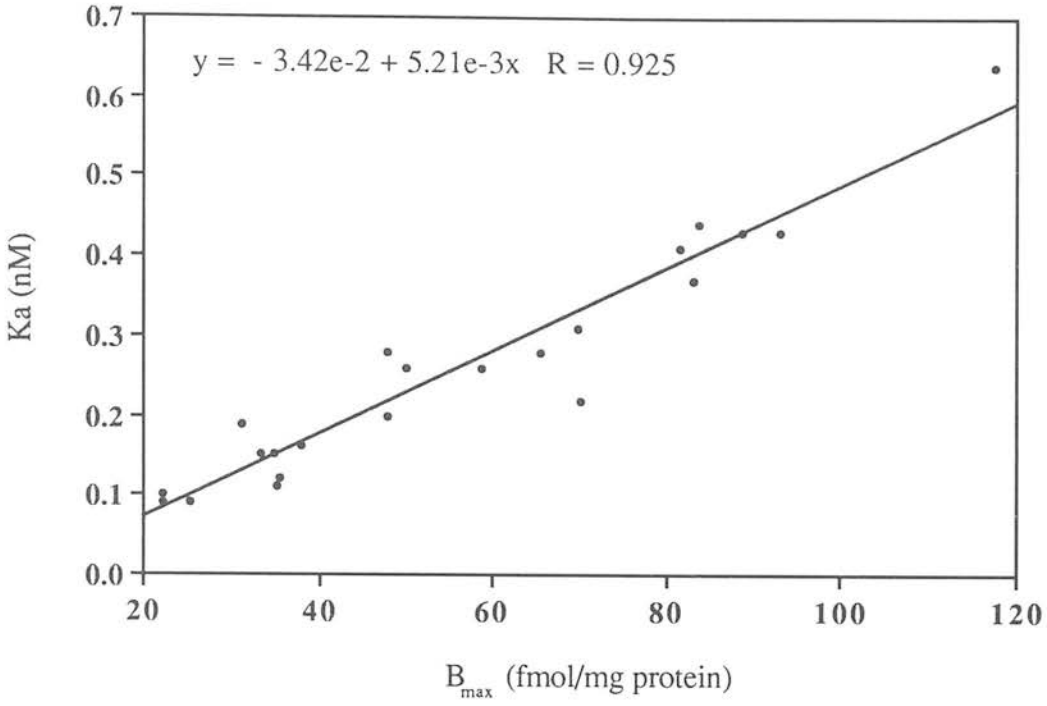


FIGURE 4.21: Correlation between K_a and B_{max} for the binding of ^{125}I-[D-Lys^6]-GnRH to pituitary glands of chickens.
Values for K_a and B_{max} (Kawashima *et al.*, 1992a) are plotted and a straight line fitted by linear regression.

4.3.3 Sex differences in the duration of GnRH-I-stimulated LH secretion

In CHAPTER 3, five explanations were considered for the difference between adult cockerels and laying hens in the duration of the plasma LH response to GnRH-I *in vivo*. First, GnRH-I may stimulate LH synthesis in pituitary tissue from laying hens but not from adult cockerels and subsequently prolong the release of LH in a second phase secretion. Second, pituitary glands of adult hens but not from adult cockerels may continue to release LH for some time after the concentration of injected GnRH-I had decreased below a stimulatory threshold ('carry over' effect). Third, a positive feedback effect involving a limited release of progesterone and GnRH-I may prolong the secretion of LH from pituitary glands of laying hens. Fourthly, pituitary glands of adult cockerels may lose their responsiveness to GnRH-I more rapidly than those from laying hens. Finally, the circulatory half-life of the endogenous forms of LH in adult chickens may be sexually differentiated. These explanations are re-considered in the light of the present results *in vitro* which allowed LH release from the pituitary gland to be studied without having to take into account the endocrine interactions of the hypothalamic-pituitary-gonadal axis, and differences in metabolic clearance rates for circulating LH.

A 3-hour stimulation of pituitary glands from laying hens with GnRH-I did not induce a second phase of LH secretion *in vitro*, which means that GnRH-I alone does not boost the rate of LH secretion to a second phase *in vivo*. Removal of the GnRH-I stimulus immediately reduced the secretion of LH from perifused pituitary glands from adult cockerels and hens. This makes it

unlikely that injection of GnRH-I induces a 'carry over' effect on LH release from pituitary glands of laying hens after the concentration of GnRH-I decreases below a stimulatory threshold. The absence of a prolonged secretion of LH in response to GnRH-I *in vitro* from pituitary glands from laying hens is consistent with the view that GnRH-I initiates a stimulatory action of increased progesterone secretion on LH release *in vivo* (see SECTION 3.3.4). It follows that a fully developed ovary has to be present to allow the prolongation of the LH response to GnRH-I. In keeping with this, adult cockerels and juvenile males and females do not show such a prolonged release of LH in response to an injection of GnRH-I *in vivo* (SECTION 3.2.2.2). The possibility cannot be excluded that the half-life of LH in plasma is sexually differentiated in adult chickens.

4.3.4 Sex differences in the profile of GnRH-I-stimulated LH secretion

The sexually differentiated profile of increased plasma LH after an injection of GnRH-I between adult cockerels and laying hens *in vivo* (CHAPTER 3) was also observed in GnRH-I-stimulated isolated pituitary glands from adult chickens *in vitro*. Two distinct phases of LH release (a spike phase followed by a plateau phase) were stimulated by GnRH-I from perifused pituitary glands from juvenile birds of both sexes and from adult cockerels. The GnRH-I-stimulated profile of LH release from pituitary tissue from laying hens was characterised by the absence of a pronounced initial spike phase. In contrast, a pronounced spike phase of LH release was observed after GnRH-I stimulation of pituitary glands from juvenile males and females. Sexual maturation of the hen is associated with a reduction in pituitary responsiveness to mGnRH *in vivo* (Bonney *et al.*, 1974; Wilson & Sharp, 1975b). The present observations are consistent with a progressive loss of the spike phase of LH release in response to GnRH-I during sexual maturation of the hen. The maturing cockerel retained a biphasic pattern of secretion *in vitro* which probably explains the similarity between the GnRH-I-stimulated profiles of plasma LH in juvenile and adult cockerels. These observations support the view that the rising concentration of plasma 17ß-oestradiol in the maturing hen contributes to the change in the GnRH-I-stimulated pattern of LH secretion from the pituitary gland on reaching adulthood (discussed in SECTION 3.3). It is probable that the sex difference in the GnRH-I-induced pattern of LH secretion from the pituitary gland *in vitro* at least partly determines the sexually differentiated profile of plasma LH in response to GnRH-I injection in adult chickens.

The mechanism involved in producing the initial phase of LH secretion in response to GnRH-I is of key interest because this feature is sexually differentiated. The descending component of the spike phase of LH release from pituitary glands from adult cockerels and both of the juvenile sexes, stimulated continuously with GnRH-I, suggests a reduction in responsiveness of the pituitary gland to GnRH-I. This is supported by the observation that repeated pulses of GnRH-I produced a succession of progressively smaller LH responses in pituitary tissue from juvenile sexes, and perhaps also the adult cockerel (FIGURE 4.4), and the magnitude of these reductions are related to the concentration of GnRH-I employed. Since the reduction in amplitude of the LH peaks was more striking in response to the higher concentration of GnRH-I, this suggests that the

reductions are not due to an artefactual decrease with increasing time in perifusion. The decreases in responsiveness may be achieved through a partial desensitisation of the pituitary gland to GnRH-I, or a depletion of the readily releaseable stores of pituitary LH.

Similar studies using pituitary tissue from chickens (King *et al.*, 1986, 1987) and turkeys *in vitro* (Guémené & Williams, 1992a) observed a progressive decrease in the magnitude of LH secretion in response to GnRH-I with no change in total pituitary LH content, and were interpreted as evidence for desensitisation. However measurements of pituitary LH content may be irrelevant because not all the pituitary LH is available for immediate release (SECTION 4.3.1). This means that the 'desensitisation' responses to GnRH-I reported previously (King *et al.*, 1986, 1987; Guémené & Williams, 1992a), are also interpretable as a reduction in the readily releaseable stores of LH.

The sexually differentiated profile of LH secretion was a characteristic response to GnRH-I but not to veratridine or increased K^+. Thus despite retaining the sex difference in magnitude of LH release, veratridine or a depolarising concentration of K^+ produced qualitatively similar *monophasic* profiles of LH secretion from pituitary tissue from both sexes of adults. Depolarising agents release LH from the same pituitary pool as GnRH (i.e. a readily releaseable pool of LH; Johnson & Mitchell, 1991) which suggests that the decline in GnRH-I-stimulated LH release from spike phase to plateau phase, is not due to depletion of LH. This deduction is consistent with the morphological observation on the distribution of LH secretory granules in the subplasmalemmal region of gonadotroph cells from adult males and females, even though there is a difference in the amount of RRP of LH in these cells. Thus there are *fewer* secretory granules in LH-gonadotroph cells of laying hens than those of adult cockerels, but the *same density* of granules in the subplasmalemmal region of the cytoplasm. This observation is inconsistent with the view that the falling phase of the spike component of LH release depends on the distribution and depletion of secretory granules in the gonadotroph cell. In other words, the spike phase is not formed by a large release of LH from a large readily releaseable store of secretory granules which depletes rapidly to allow the rate of release to decrease to a plateau phase of secretion.

Alternative explanations for the phasic nature of the GnRH-I-stimulated release of LH *in vitro* are that there are more than one functional class of LH-gonadotroph cell responding to GnRH-I which contribute to the two phases of secretion, or that each phase involves different mechanisms of LH secretion from a single class of gonadotroph cell (see CHAPTER 7). The former possibility was not investigated because only one cell-type is reported to be responsive to GnRH-I in the chicken pituitary gland (King *et al.*, 1987).

When pituitary tissues from adult cockerels and hens were perifused with GnRH-I for 3-hours, a decrease in LH release from pituitary glands from males was seen during the plateau phase, whereas no decrease in responsiveness to GnRH-I was seen in the female tissues (FIGURE 4.7). At the end of stimulation with GnRH-I, a maximal dose of veratridine released a concentration of

LH from pituitary tissue from both sexes, not different from that immediately prior to withdrawal of GnRH-I. Thus regardless of whether the spike phase of secretion in adult cockerels and juveniles represents desensitisation to GnRH-I or depletion of readily releaseable LH, the pituitary gland of the laying hen shows neither effect. Two conclusions are drawn from this observation. Firstly it suggests that the anterior pituitary gland of laying hens does not easily lose its responsiveness to GnRH-I. This finding is consistent with observations *in vivo* where constant or frequent exposure to high doses of GnRH over prolonged periods (Dickerman & Bahr, 1989; Tilbrook *et al.*, 1992) rather than daily injections of GnRH analogues (Sterling & Sharp, 1984), are required to depress reproductive function. Secondly, it suggests that the anterior pituitary gland of adult cockerels cannot maintain a constant rate of LH secretion in response to continuous exposure to GnRH-I due to a depletion of releaseable stores of LH, whereas a sustainable rate of LH release under the same conditions is achieved from pituitary tissue from laying hens. This indicates that gonadotroph cells from laying hens replenish their releaseable stores of LH at a rate similar to the secretion of LH. LH is recruited to replenish the RRP stores from a 'storage' pool or is newly synthesised (Bremner & Paulsen, 1974; Hoff *et al.*, 1977; Adams & Nett, 1979; Naor *et al.*, 1982). In contrast, prolonged stimulation with GnRH-I may result in an imbalance between release and repletion of LH in the gonadotroph cells of the adult cockerel, eventually decreasing the release of LH. Therefore, there could be a sex difference in the processes of LH synthesis or translocation of secretory granules in the gonadotroph cells of adult chickens, which results in the sexually differentiated profile of LH secretion in response to GnRH-I *in vitro*.

In mice, the translocation of LH secretory granules is mediated by microfilaments which mobilise granules from a storage site of the cell, to a 'functional pool' which is available for immediate release (Lewis *et al.*, 1985, 1986; Ravindra & Grosvenor, 1990). This margination of secretory granules towards the subplasmalemmal pool of LH is important in the priming effect of GnRH by enhancing its capacity to release LH during the rodent preovulatory surge of LH (Lewis *et al.*, 1985, 1986). Although repeated exposures to GnRH-I did not enhance the secretion of LH from pituitary tissue from laying hens, GnRH-I may activate processes which replenish the RRP of LH such that the gonadotroph cells can maintain a sustained release of LH. This might be important for maintaining the preovulatory surge of LH in laying hens over several hours.

4.3.5 Sex differences in the distribution of secretory granules in GnRH-I-stimulated gonadotroph cells of the adult chicken

The sexually differentiated profile of LH secretion from adult pituitary glands may be related to GnRH-I-induced changes in the number and distribution of secretory granules in gonadotroph cells (see SECTION 4.2.4). Two-minutes after injection of GnRH-I, the increase in number of secretory granules observed in the 'readily releaseable region' of cytoplasm in gonadotroph cells of both sexes, was associated with an increased secretion of LH from the pituitary gland, and increased concentrations of plasma LH. The number of secretory granules in the subplasmalemmal region remained elevated at 30-minutes after injection in both sexes. This is consistent with the

sustained release of LH in laying hens at this time, but does not correlate with the declining concentration of plasma LH in adult cockerels. It is possible that by 30-minutes after injection, the concentration of GnRH-I decreases to levels insufficient to stimulate LH release in both sexes, but in laying hens, the prolonged LH response is maintained by a stimulatory action of progesterone on LH release (see SECTION 3.3.4). The observations in the male indicate that the GnRH-I-stimulated concentration of plasma LH *in vivo* and the spike of LH secretion *in vitro* do not fall because of depletion of secretory granules in the subplasmalemma of gonadotroph cells.

4.3.6 Lipid-containing cells in the pituitary gland of the adult cockerel

An 'LH-gonadotroph-like' cell which has been not described previously was identified during the course of ultrastructural studies of the anterior pituitary gland from adult cockerels. These cells were typified firstly by very large electron-dense structures in the endoplasmic reticulum, and secondly these cells were only found in pituitary glands from adult cockerels, but not in those from age-matched hens. The lipid nature of these bodies was demonstrated by their osmiophilic property in cryostat sections (Hayat, 1970; FIGURE 4.18), and by the observation of 'wave-like' striations across the material (FIGURE 4.13), typical of lipid in transmission electron microscopy (Hayat, 1970). These structures were not bounded by an immediate membrane and therefore, were probably not lysosomes. However the occasional association of this lipid material with acid phosphatase-positive staining and delimitation by a membrane (FIGURE 4.15) suggests degradation of this material in some cells.

In view of the sex difference in the distribution of these inclusions, it is of interest to note that similar inclusions have been reported in the pituitary gland of quail with *inactive* ovaries (Mikami *et al.*, 1975). However these cells are absent in sexually *active* female quail (Mikami *et al.*, 1975), White-crowned Sparrows (*Zonotrichia leucophrys gambelii*; Mikami *et al.*, 1969), and laying hens (Mikami, 1973; Tai, 1976; Tai & Chadwick, 1977). Furthermore, preliminary observations indicate that pituitary glands from juvenile (7-week old) male and female chickens do not possess these lipid-containing cells. The presence of these cells in the pituitary gland of sexually inactive female birds and sexually mature cockerels, but not in those of sexually active females, may be of significance in avian reproduction. The question as to what function these cells serve is difficult to surmise from the present study. However, the cells resemble LH-gonadotroph cells (TABLE 4.5) and may be a transitional type of LH-cell.

4.4 SUMMARY

The sex differences in the responsiveness and sensitivity of the pituitary gland to GnRH-I observed *in vivo* were shown to be essentially the same *in vitro*. The GnRH-I-induced magnitude and profile of LH secretion, but not the duration of the response, are therefore a function of the pituitary gland. GnRH-I released less LH from pituitary glands from laying hens because of a

lower K^+-releaseable pool of LH, and fewer secretory granules in the subplasmalemmal region of the cytoplasm of LH-gonadotroph cells compared with the corresponding values for pituitary glands from adult cockerels. This smaller LH response of pituitary glands from laying hens was not due to a sex difference in the proportion of LH-gonadotroph cells in pituitary glands of adult chickens, although these cells were smaller in laying hens. Instead, the small LH response is probably related to a loss of the GnRH-I-stimulated spike phase of LH secretion from the pituitary gland of the hen during sexual maturation, but which is retained in the adult cockerel.

The sexually differentiated pattern of LH release from the adult pituitary gland was similar to the profile of plasma LH *in vivo* after administration of GnRH-I. The descending portion of the spike phase was not due to depletion of readily releaseable stores of LH in the pituitary gland, however depletion of LH stores did develop after a prolonged stimulation of pituitary glands from adult cockerels with GnRH-I. An alternative hypothesis is that the sharp decrease in LH secretion is due to desensitisation of the pituitary gland of adult cockerels to GnRH-I. This possibility could not be established clearly from the present study. There was no evidence in these experiments for depletion of the releaseable stores of LH or pituitary desensitisation by GnRH-I in laying hens. A more potent and more sustained stimulation by GnRH-I may be required for these effects to be expressed in adult hens. This suggests that gonadotroph cells of the adult female can support a low but sustained release of LH in response to a continuous stimulation by GnRH-I without a loss of responsiveness, and possibly from a store of LH which is replenished at a rate equivalent to the rate of LH release

The sexually differentiated duration of elevated LH in response to GnRH-I was not reproduced *in vitro*. It is concluded that extrapituitary factors are important in the prolonged duration of LH release in response to GnRH-I. This may be due to a sex difference in the circulatory half-life of endogenous LH in adult chickens, or the presence of a stimulatory effect of progesterone in laying hens.

The attempts to measure GnRH-receptors were unsuccessful. It therefore remains to be determined whether the sexually differentiated sensitivity to GnRH-I is due to a difference in the GnRH-receptor characteristics of pituitary glands of adult male and female chickens.

Finally, an anterior pituitary cell which contained large amounts of lipid was identified in adult cockerels but not in laying hens. The physiological significance of this observation requires further investigation.

5 The Effects of Oestradiol Benzoate on the Hypothalamic-Pituitary Axis of the Adult Intact Cockerel

5.1 Introduction

It has been suggested (Wilson & Sharp, 1975b; Sharp *et al.*, 1987; Wilson *et al.*, 1989) that the sex difference in the LH response to GnRH-I is caused by the increase in concentration of plasma 17ß-oestradiol resulting from the growth of the ovary (Peterson & Webster, 1974; Senior, 1974). There is both physiological and pharmacological evidence for an inhibitory action of 17ß-oestradiol on the hypothalamic-pituitary complex. First, the depression in plasma 17ß-oestradiol resulting from regression of the ovaries of incubating and brooding bantam hens, increases the LH responsiveness to injections of GnRH (Sharp & Lea, 1981). Second, ovariectomy increases the concentration of baseline LH in the chicken (Sharp, 1975; Wilson & Sharp, 1975c), and increases the magnitude of the LH response to GnRH (Wilson, 1975). Third, injections of 17ß-oestradiol reduce the concentration of plasma LH in laying hens (Wilson & Sharp, 1976a, 1976b). Fourthly, treatment of laying hens with the anti-oestrogen tamoxifen blocks the inhibitory effect of 17ß-oestradiol on the baseline concentration of LH and increases pituitary responsiveness to injections of GnRH (Wilson & Cunningham, 1981). Oestrogen can also act directly on the pituitary gland of sexually immature chickens to suppress the LH response to GnRH *in vitro* (Bonney & Cunningham, 1977d; Luck & Scanes, 1980; King *et al.*, 1989). Finally, the GnRH-induced release of LH from pituitary cells of juvenile chickens is reduced by treatment with 17ß-oestradiol *in vitro* (SECTION 1.5.2).

Unlike in hens, sexual development in cockerels is associated with an increase in the concentration of plasma testosterone, rather than of 17ß-oestradiol (Tanabe *et al.*, 1979, 1981; Knight, 1983; Stansfield & Cunningham, 1988). However, testosterone is thought to be aromatised to 17ß-oestradiol by the hypothalamus to exert an inhibitory feedback effect on GnRH-I release (Wilson *et al.*, 1983). This means that the action of testosterone is mediated through 17ß-oestradiol. However the high concentration of testosterone in the adult male does not produce the changes in the LH response to GnRH-I characteristic of the laying hen (Sharp *et al.*, 1987; CHAPTER 3). The present study involved administering 17ß-oestradiol to adult cockerels in order to simulate the high concentrations of plasma 17ß-oestradiol in laying hens in an attempt to 'feminise' the hypothalamic-pituitary control of LH.

5.2 RESULTS

5.2.1 Effect of oestradiol benzoate on the GnRH-I-stimulated LH response

The baseline concentration of plasma LH decreased with increasing concentration of plasma 17ß-oestradiol within 24-hours after injection of oestradiol benzoate (FIGURE 5.1). The concentration of plasma 17ß-oestradiol which reduced the control concentration of LH to half ΔLH (IC_{50}) was 400 pg/ml (or 1.5 nM 17ß-oestradiol); equivalent to a dose of approximately 0.01 mg oestradiol benzoate/kg body weight.

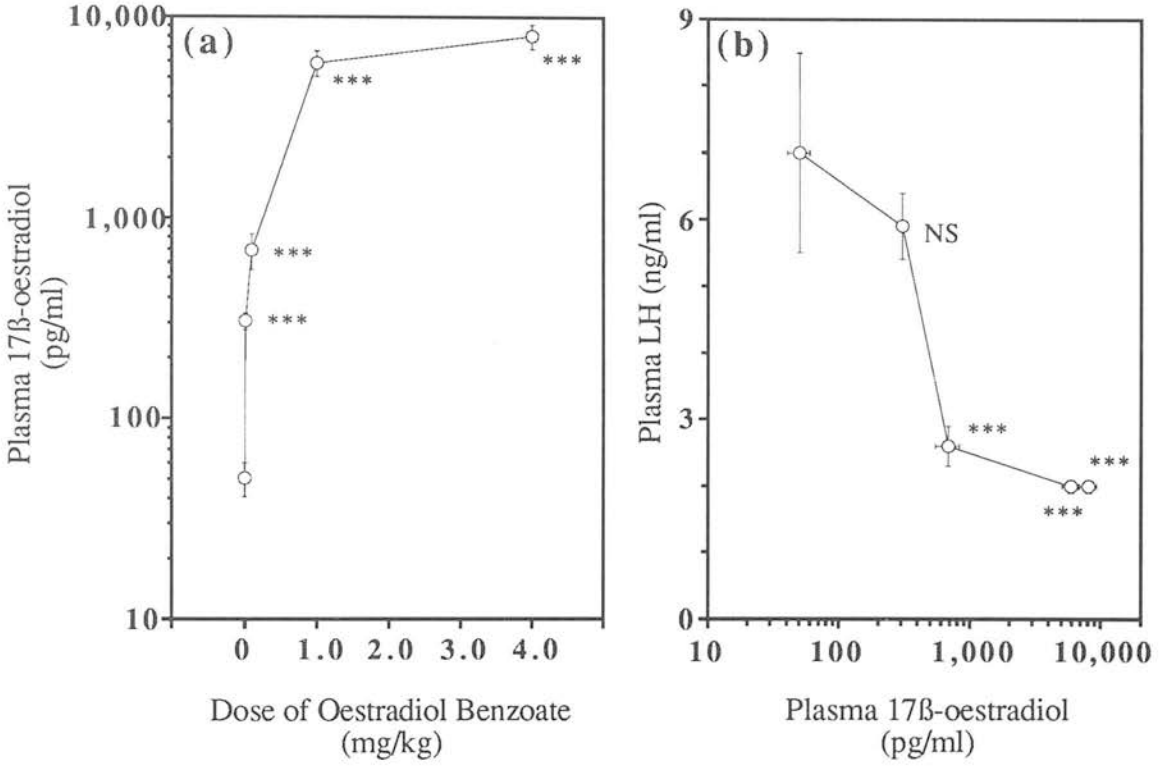


FIGURE 5.1: Relationship between dose of oestradiol benzoate, the concentration of plasma 17ß-oestradiol and the resting concentration of plasma LH in adult cockerels. Experiment 1. Cockerels were given a single intramuscular injection of oil, or 0.01, 0.1, 1.0 or 4.0 mg oestradiol benzoate/kg body weight (n = 8 per group). Concentrations of (a) plasma 17ß-oestradiol and (b) plasma LH were determined 24-h later. All doses of oestradiol benzoate increased (***P<0.001) the concentration of plasma 17ß-oestradiol compared with the control. (b) NS = not significantly different, ***P<0.001 compared with the concentration of LH of cockerels injected with vehicle.

GnRH-I increased (P<0.05) the concentration of plasma LH in all treatment groups (FIGURE 5.2), however the magnitude of the peak of plasma LH decreased with increasing dose of oestradiol benzoate (P<0.001 at doses ≥ 0.1 mg oestradiol benzoate/kg). There were no differences in the profile of plasma LH between cockerels treated with 1 or 4 mg oestradiol benzoate/kg. A reduction in the LH response was also reflected by the total amount of LH released (area-under-the-curve; FIGURE 5.3). LH concentrations returned to their control levels within 60 - 120-minutes of GnRH-I injection. The time for the concentration of plasma LH to decline to half of its GnRH-I-stimulated peak, was 17.5 ± 3.0, 16.8 ± 2.5, 18.0 ± 3.1, 17.5 ± 3.5 and 21.1 ±

4.0 minutes for respectively 0, 0.01, 1 and 4 mg oestradiol benzoate/kg-injected birds (not significantly different from control; n = 8; linear regression analysis; correlation coefficients = 0.894 - 0.998).

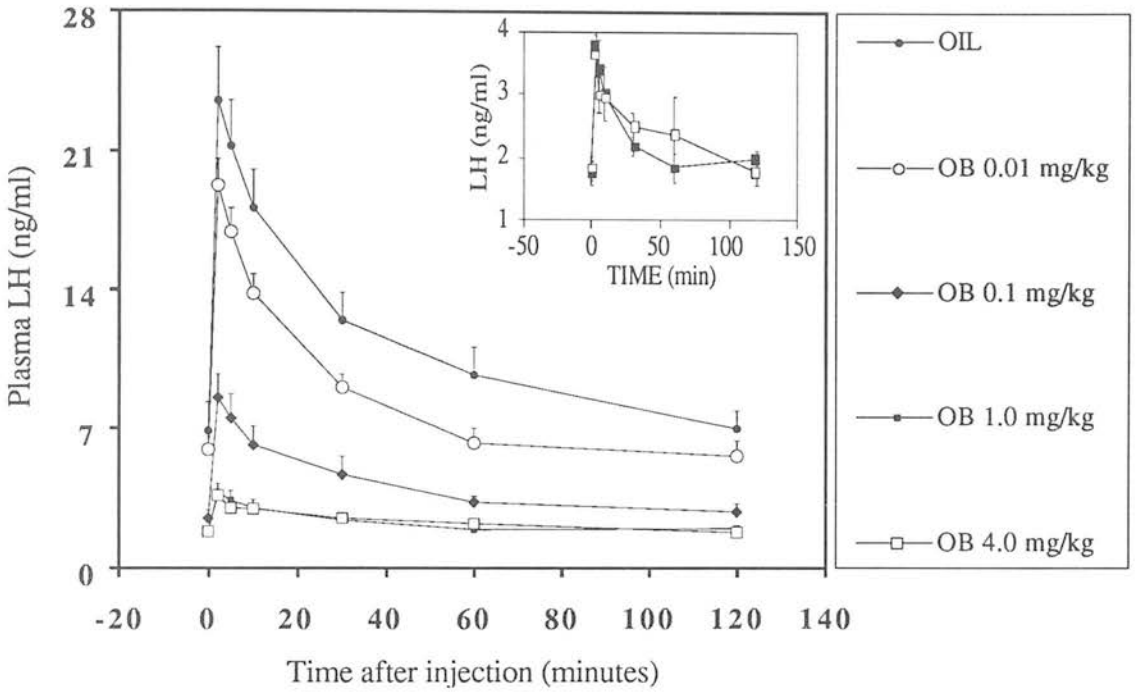


FIGURE 5.2: Effect of oestradiol benzoate treatment on the LH response to GnRH-I in adult cockerels.
Each bird was given a single intravenous injection of 0.5 µg GnRH-I/kg body weight 24-h after oestradiol benzoate (OB) injection. Blood samples were collected for up to 120-min (n = 8). INSET shows the similarity in the profiles of plasma LH between the 1 and the 4 mg oestradiol benzoate/kg groups.

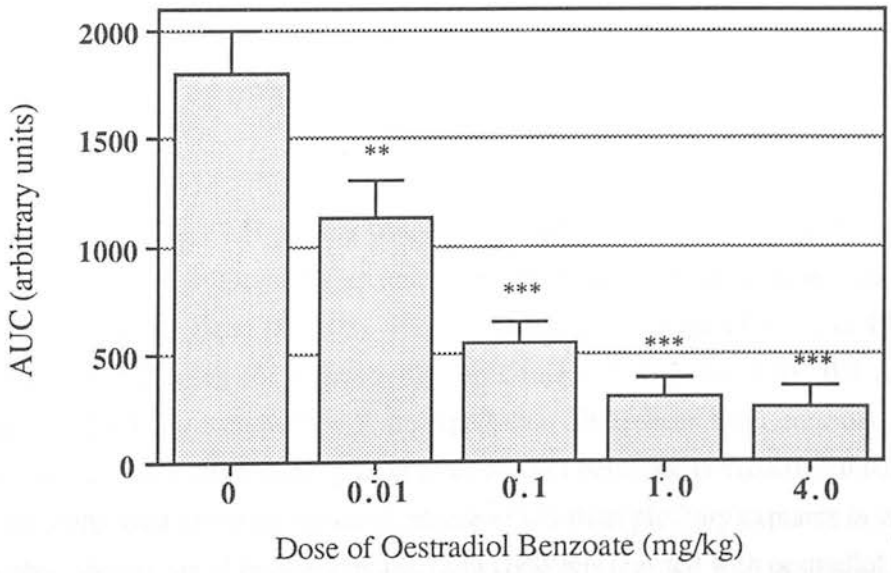


FIGURE 5.3: Effect of oestradiol benzoate in adult cockerels on the relative amount of LH released in response to a single injection of 0.5 µg GnRH-I/kg.
See FIGURE 5.2 for legend. The area-under-the-curve (AUC) of plasma LH after GnRH-I injection was calculated by MUNRO™ (see SECTION 2.7). **P<0.01, ***P<0.001 compared with oil-injected control; n = 8.

5.2.2 Effect of oestradiol benzoate on pituitary LH and hypothalamic GnRH-I and GnRH-II content

The 4 mg oestradiol benzoate/kg treatment group was omitted from a subsequent experiment because there was no difference in the concentrations of plasma LH or 17ß-oestradiol between the two highest dose groups (FIGURE 5.1). As in Experiment 1, injection of oestradiol benzoate reduced the baseline concentration of plasma LH 24-hours after treatment ($P<0.01$ at ≥ 0.1 mg/kg; FIGURE 5.4).

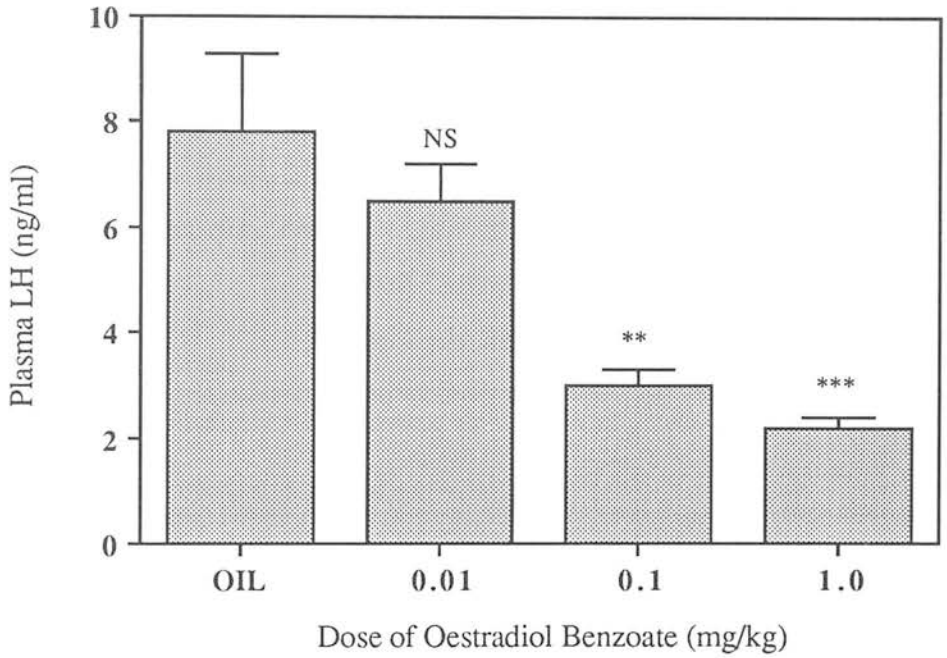


FIGURE 5.4: Effect of oestradiol benzoate on baseline concentration of plasma LH. Experiment 2. A blood sample was collected for LH measurement 24-h after a single intramuscular injection of oil or oestradiol benzoate. NS = not significantly different, **P<0.01, ***P<0.001 compared with oil-injected control; n = 8 per group.

The concentration of pituitary LH per mg tissue weight was not affected by steroid treatment (FIGURE 5.5), however significant differences were seen when the data were expressed as amount of LH per pituitary gland ($P<0.05$). The pituitary weights were 15.8 ± 1.6, 13.7 ± 0.6, 14.2 ± 0.8 and 13.8 ± 0.6 mg for respectively birds injected with oil, 0.01, 0.1 and 1 mg oestradiol benzoate/kg body weight (n = 8; no significant differences from control). The K^+-releaseable LH decreased with increasing dose of oestradiol benzoate ($P<0.001$; FIGURE 5.6), but there was no significant effect on the basal release of LH from pituitary explants *in vitro*. The readily releaseable pool of LH of pituitary tissues from cockerels injected with oestradiol benzoate was significantly less ($P<0.001$) than that from oil-injected controls (90.4 ± 8.0 ng LH/mg tissue weight, compared with 29.3 ± 4.9, 29.2 ± 5.5, and 22.8 ± 5.0 ng LH/mg tissue weight for respectively 0.01, 0.1 and 1.0 mg oestradiol benzoate/kg body weight).

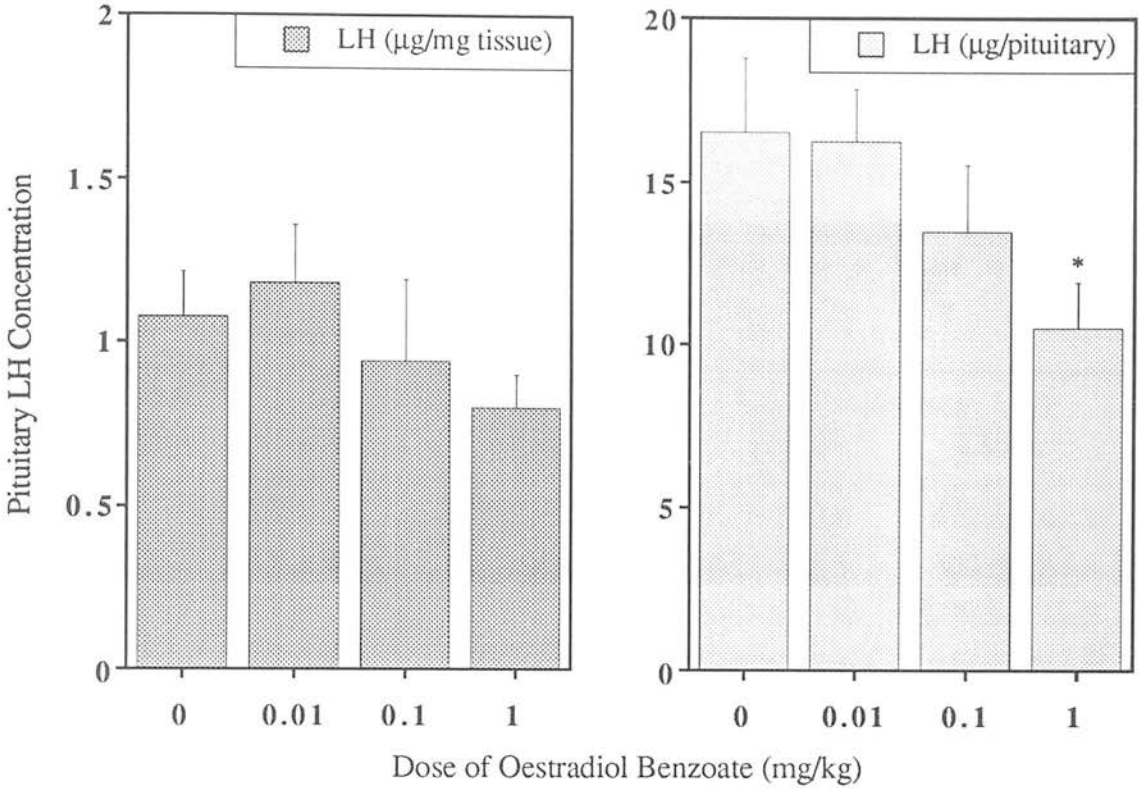


FIGURE 5.5: Effect of oestradiol benzoate on the pituitary content and concentration of LH in adult cockerels.
Twenty-four hours after an injection of oestradiol benzoate, the pituitary gland was removed, longitudinally bisected and weighed. Total LH was measured from one hemipituitary gland of each bird (n = 8). Concentration of pituitary LH is expressed as µg/mg tissue or µg/pituitary gland. The other hemipituitary gland was incubated in Ringer for 30-min (Basal LH) then in 60 mM K^+ Ringer for 30-min (see FIGURE 5.6). *P<0.05 compared with pituitary tissue from oil-injected control. Note difference scale on y-axes.

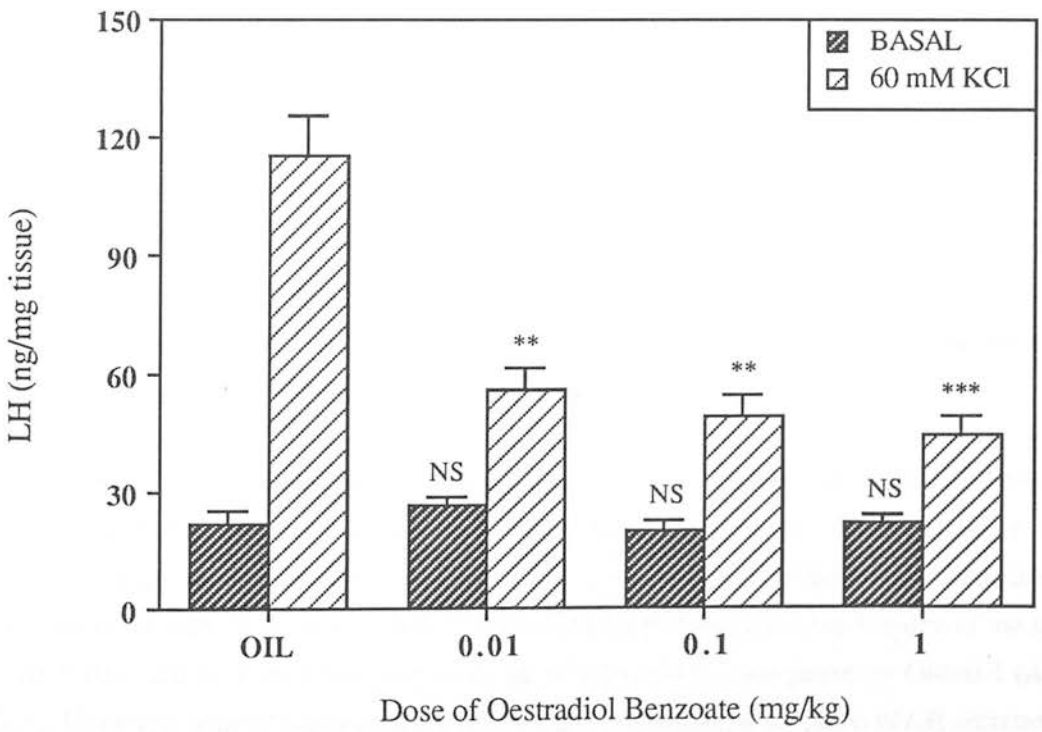


FIGURE 5.6: Basal and K^+-releaseable LH from pituitary glands of the adult cockerel 24-hours after a single injection of oestradiol benzoate.
See FIGURE 5.5 for legend. Readily releaseable pool of LH = K^+-releaseable LH minus basal LH; values given as mean ± sem. NS = not significantly different, **P<0.01, ***P<0.001 compared with the control.

A dose-related effect was not established between oestradiol benzoate and the contents of GnRH-I and GnRH-II in the MBH and POA (TABLE 5.1), and only 0.1 mg oestradiol benzoate/kg significantly ($P<0.05$) reduced the content of GnRH-II.

TABLE 5.1: The contents of GnRH-I and GnRH-II in the mediobasal hypothalamus (MBH) and preoptic area (POA) of adult cockerels 24-h after a single injection of oestradiol benzoate.

Dose of OB (mg/kg BW)	GnRH-I/tissue (ng)		GnRH-II/tissue (pg)	
	MBH	POA	MBH	POA
0	3.9 ± 0.8	1.2 ± 0.1	214.2 ± 38.0	522.0 ± 61.1
0.01	2.7 ± 0.8	1.1 ± 0.2	189.5 ± 23.5	405.0 ± 44.8
0.1	4.7 ± 1.4	0.9 ± 0.1	184.9 ± 21.0	*289.6 ± 69.5
1.0	2.2 ± 0.7	1.0 ± 0.1	215.1 ± 27.4	587.5 ± 87.8

*$P<0.05$ compared with the amount in tissue from oil-injected controls (n = 8).

5.2.3 The direct effect of treating pituitary glands from adult cockerels with 17ß-oestradiol on the LH response to GnRH-I *in vitro*

The reduction in gonadotroph function in adult cockerels injected with increasing doses of oestradiol benzoate may be due to a direct effect of 17ß-oestradiol on the pituitary gland, and an indirect effect on the synthesis and release of GnRH-I from the hypothalamus. To determine whether 17ß-oestradiol acts directly on the pituitary gland, pituitary tissue from adult cockerels were incubated for 24-hours with 17ß-oestradiol *in vitro*. The doses of 17ß-oestradiol were chosen from calculations made from the circulating levels of 17ß-oestradiol achieved in Experiment 1 (SECTION 5.2.1). A circulating concentration of 1.5 nM 17ß-oestradiol reduced the concentration of plasma LH to half ΔLH in FIGURE 5.1. The maximum concentration of plasma 17ß-oestradiol achieved 24-hours after injection of oestradiol benzoate was 33 nM.

Treatment with 1000 nM 17ß-oestradiol significantly reduced the baseline release of LH compared with the control ($P<0.001$; FIGURE 5.7, TABLE 5.2), but not with lower doses of 17ß-oestradiol. GnRH-I stimulated a biphasic release of LH from pituitary tissues from all treatment groups. Treatment with all doses of 17ß-oestradiol significantly increased the duration of the spike phase ($P<0.05$), and increased the plateau phase of LH release stimulated by GnRH-I (ΔLH; $P<0.001$). However, none of the treatments affected the magnitude of the spike of LH secretion or the total amount of LH released (AUC). The incremental change in the plateau phase of LH secretion relative to that of the spike phase was reduced ($P<0.05$) by treatment with 17ß-oestradiol.

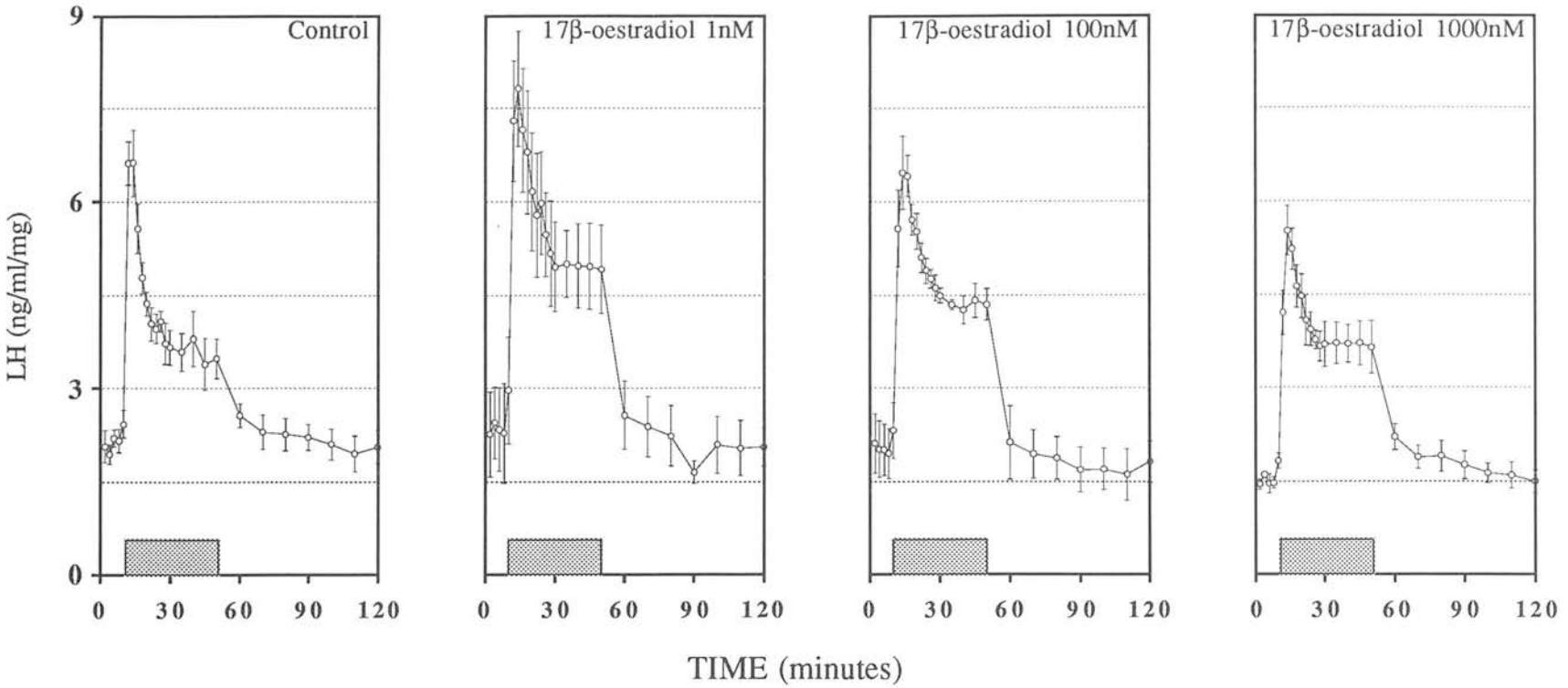


FIGURE 5.7: Baseline and GnRH-I-induced LH secretion from perifused pituitary tissue of the adult cockerel incubated *in vitro* for 24-hours in the presence of 17ß-oestradiol.
Quartered pituitary pieces from adult cockerels were incubated for 24-h with vehicle, 1 nM, 100 nM or 1000 nM 17ß-oestradiol (n = 4 per dose), and then perifused in the same concentration of 17ß-oestradiol. GnRH-I (100 nM) was added to the perifusion medium for 40-min (grey bar).

TABLE 5.2: An analysis of baseline and GnRH-I-induced LH secretion from perifused adult cockerel pituitary tissue incubated for 24-hours in the presence of 17ß-oestradiol *in vitro*.

Treatment	Basal LH (ng/ml/mg)	Spike LH (ng/ml/mg)	Spike ΔLH (ng/ml/mg)	Spike Duration (minutes)	Plateau LH (ng/ml/mg)	Plateau ΔLH (ng/ml/mg)	AUC LH (ng/ml/mg)	AUC ΔLH (ng/ml/mg)	Spike ΔLH -to-Plateau ΔLH Ratio
Control	2.09 ± 0.09	6.62 ± 0.53	4.53 ± 0.50	19.80 ± 0.74	3.57 ± 0.12	1.54 ± 0.07	354 ± 29	103 ± 10	2.94 ± 0.33
1 nM E_2	2.33 ± 0.31	7.81 ± 0.92	5.43 ± 0.43	25.95 ± 1.73*	5.04 ± 0.25*	2.71 ± 0.10***	427 ± 72	159 ± 24	2.00 ± 0.17*
100 nM E_2	2.01 ± 0.16	6.46 ± 0.59	4.44 ± 0.75	25.45 ± 1.16*	4.42 ± 0.08*	2.40 ± 0.14***	363 ± 37	120 ± 28	1.85 ± 0.27*
1000 nM E_2	1.50 ± 0.05***	5.52 ± 0.40	4.03 ± 0.47	22.88 ± 0.72*	3.64 ± 0.12	2.16 ± 0.14***	321 ± 27	115 ± 23	1.87 ± 0.22*

See FIGURE 5.7 for legend. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with control response.

Spike LH: maximum concentration of LH after the onset of GnRH-I perifusion

Spike ΔLH: maximum incremental change in concentration of LH after the onset of GnRH-I perifusion

Spike Duration: intersection of tangents drawn to the plateau phase and the declining component of the spike phase

Plateau LH: mean concentration of LH during the plateau phase of LH secretion

Plateau ΔLH: mean incremental change in concentration of LH during the plateau phase of LH secretion

AUC LH: total release of LH over the experimental period

AUC ΔLH: the total release of LH stimulated by GnRH-I (basal LH subtracted)

5.3 Discussion

These results show a difference between the effects of oestrogen-treatment *in vivo* and *in vitro* on the release of LH from anterior pituitary glands of adult cockerels. Injection of oestradiol benzoate depressed the resting concentration of plasma LH in adult cockerels (FIGURES 5.1 and 5.4), but did not affect the basal release of LH *in vitro* from pituitary glands from these animals (FIGURE 5.6). Further, the baseline release of LH from isolated pituitary tissue treated directly with 17ß-oestradiol *in vitro* was reduced at only the highest dose of 17ß-oestradiol employed (FIGURE 5.7). These observations indicate that 17ß-oestradiol exerts a more pronounced effect on the resting concentration of LH *in vivo* than *in vitro*.

If 17ß-oestradiol targets only the pituitary gland to inhibit LH release, then a decrease in the baseline concentration of LH would be seen *in vivo* and *in vitro*. However, a reduction was clearly seen *in vivo* but only at the highest dose of 17ß-oestradiol *in vitro*. It is therefore probable that 17ß-oestradiol acts in part at the hypothalamic level in adult cockerels to reduce the release of GnRH-I. In keeping with this, castration of juvenile cockerels increases the content and *in vitro* release of hypothalamic GnRH-I (Knight *et al.*, 1983; Wilson *et al.*, 1983, 1990b; Lal *et al.*, 1990; Sharp *et al.*, 1990), and this effect is reversed by supplements of 17ß-oestradiol (Wilson *et al.*, 1990b). However, doses of oestradiol benzoate which reduce the GnRH-I content of the posterior hypothalamus of *juvenile* cockerels (Wilson *et al.*, 1990b), had no effect on the contents of GnRH-I or GnRH-II in the MBH or the POA of *adult* intact cockerels. This discrepancy apparently indicates an age-related difference in the oestrogen-sensitivity of GnRH-I-containing neurones. This could be because any change in GnRH-I and GnRH-II contents is proportionately too small to detect against the higher contents in adult hypothalamic tissues compared with those of juvenile males (SECTION 3.2.1.1). This would mean that steady-state measurements of GnRH-I and GnRH-II in large blocks of brain tissue are not sufficiently sensitive to detect subtle changes in hypothalamic function.

As observed in sexually immature cockerels (Wilson *et al.*, 1983, 1990b), treatment of adult cockerels with oestradiol benzoate *in vivo* reduced both the peak of plasma LH and the amount of LH released (AUC) in response to GnRH-I. These effects of oestradiol benzoate indicate either a reduction in pituitary responsiveness or a reduction in its sensitivity to GnRH-I. The single dose-level of GnRH-I used in this study (0.5 µg/kg; a maximally stimulatory dose in the adult cockerel; TABLE 3.4) could not distinguish between these two explanations (however see CHAPTER 6). However, the magnitude of the LH response to injection of a maximally stimulatory dose of GnRH is increased by blocking the effects of the high concentration of 17ß-oestradiol in laying hens with tamoxifen (Wilson & Cunningham, 1981). This indicates that a rise in the concentration of circulating 17ß-oestradiol will reduce the pituitary *responsiveness* to GnRH-I.

It is difficult to discern whether the pituitary effects of 17ß-oestradiol *in vivo* are due to a direct action on the pituitary gland itself, or are secondary to a change in hypothalamic function.

Oestrogen did not affect the total release of LH in response to GnRH-I when pituitary glands from adult cockerels were treated with 17ß-oestradiol *in vitro*, but gonadal steroids directly suppress the LH response to GnRH of pituitary cells from sexually immature chickens *in vitro* (Bonney & Cunningham, 1977d; Luck & Scanes, 1980; King *et al.*, 1989). The suppression of LH secretion from pituitary tissue from adult cockerels may require a longer period of treatment with 17ß-oestradiol in order to express a marked change. Alternatively, the absence of perfusion *through* the tissue could mean that pituitary pieces incubated with 17ß-oestradiol *in vitro* may not be penetrated by 17ß-oestradiol to the same extent as pituitary glands in the whole animal. However, this experimental protocol is similar to a technique which demonstrated increased secretion of prolactin but not of growth hormone from hemipituitary glands from juvenile chickens after 3 or 20-hour incubations with 17ß-oestradiol *in vitro* (Hall *et al.*, 1984). This suggests that the weak effects of 17ß-oestradiol on the magnitude of the GnRH-I-stimulated release of LH may not to be due to inadequacies of the technique. Consequently these observations suggest that gonadotroph cells of adult cockerels could be less sensitive to 17ß-oestradiol than those of juvenile cockerels. It may therefore prove more fruitful to study the effect of 17ß-oestradiol on the LH responses of pituitary tissue from juvenile chickens in response to GnRH-I (see CHAPTER 6). The decrease in pituitary function induced by 17ß-oestradiol *in vivo* could therefore result in part by a change in hypothalamic function and by a direct effect on gonadotroph cells.

The depressed responsiveness of oestrogen-treated adult cockerels to GnRH-I injection could be due to the reduced amount of LH available for release in the pituitary gland. The possibility that this reduction is through an inhibition of LH synthesis is supported by the depressed content of pituitary LHß-mRNA in juvenile hens treated with oestradiol benzoate (Kallmeier *et al.*, 1991). In the rat, ovariectomy increases and 17ß-oestradiol suppresses the levels of gonadotrophin subunit mRNA in the pituitary gland (reviewed by Marshall *et al.*, 1990). Since changes in the steady-state amount of LHß-mRNA only indicates the balance between its formation and degradation, an elevated level of LHß-mRNA suggests either an increase in LHß-gene expression or a reduction in degradation of the gene transcript. The size of α-subunit and LHß-mRNAs in the rat pituitary gland increases after ovariectomy, by increasing the extent of polyadenylation of the transcripts (Weiss *et al.*, 1992) which extends the cellular half-life of mRNA (Bernstein *et al.*, 1989). This effect of ovariectomy depends on changes in GnRH secretion because direct treatment of rat pituitary cells with GnRH also increases the size of α-subunit and LHß-mRNAs by polyadenylating the transcripts (Weiss *et al.*, 1992). Furthermore, 17ß-oestradiol reduces the pulse frequency of GnRH release from the hypothalamus in the ovariectomised ewe (Karsch *et al.*, 1987), and reduces the synthesis and release of GnRH in rats (Zoeller *et al.*, 1988; Toranzo *et al.*, 1989; Radovick *et al.*, 1991). However, the content of LHß-mRNA in rat pituitary cells is also reported to be reduced by 17ß-oestradiol *in vitro* (Shupnik *et al.*, 1989; Mercer *et al.*, 1990), which indicates a direct suppressive effect on LH synthesis. The combined direct and indirect effects of 17ß-oestradiol on the pituitary gland reduces the LH secretory and synthetic activity of gonadotroph cells in the rat (Lalloz *et al.*, 1988; Dalkin *et al.*, 1989; Haisenleder *et al.*, 1988, 1991). The depressed level of pituitary LHß-mRNA by treatment with 17ß-oestradiol in the

chicken (Kallmeier *et al.*, 1991) therefore indicates increased degradation of LHß-mRNA and/or reduced LHß-gene expression. This effect of 17ß-oestradiol could be mediated through a direct and indirect effect on the pituitary gland to reduce LH synthesis.

The episodic pattern of plasma LH (Wilson & Sharp, 1975c) is thought to inducate a similar pattern of GnRH-I release (see SECTION 1.3.4). The magnitude of LH release in response to perifusion with 100 nM GnRH-I was lower in pituitary tissue deprived of endogenous GnRH-I for 24-hours *in vitro* (spike = 4.5 ± 0.5, plateau = 1.5 ± 0.1 ng/ml/mg; FIGURE 5.7) compared with the response of freshly collected tissue (spike = 49.3 ± 5.0, plateau = 16.3 ± 3.1 ng/ml/mg; FIGURE 4.3; $P<0.001$ compared with respective responses of cultured tissue in both cases). This could mean that the pituitary gland requires an environment of GnRH-I to maintain the content of LH and its responsiveness to GnRH-I. However another reason for the small release of LH from pituitary pieces after 24-hours *in vitro* could be due to development of tissue necrosis. Necrosis at the core of blocks of frog pituitary tissue is evident after 24-hours in culture (Porter & Licht, 1985).

Oestrogen did not affect the total duration of the LH response to GnRH-I *in vivo* or *in vitro*. This was illustrated *in vitro* by the abrupt fall in LH secretion from perifused pituitary tissues on withdrawal of GnRH-I (FIGURE 5.7). Also, stimulation of the oestrogen-conditioned pituitary gland of the adult cockerel with GnRH-I did not reveal a laying hen-type LH response. Instead, the profile of secretion remained biphasic in character. The GnRH-I-stimulated prolonged LH response of laying hens may require factors other than 17ß-oestradiol for this effect. It was suggested that the presence of a stimulatory action of progesterone could be important in this response or that there is a sex difference in the circulatory half-life of LH in adult chickens (SECTION 3.3.4).

Treatment of pituitary glands with 17ß-oestradiol *in vitro* altered the profile of LH secretion in response to GnRH-I. Thus, the concentration of LH during the plateau phase of secretion was increased by 17ß-oestradiol without affecting the magnitude of the initial peak of release. However a longer period of treatment with 17ß-oestradiol may be required because of the apparent downward trend in the spike phase of LH secretion (FIGURE 5.7, TABLE 5.2). The effect of 24-hours of treatment with 17ß-oestradiol on the GnRH-I-stimulated spike and plateau phases of LH secretion has been studied in cultures of pituitary cells from juvenile chickens (King *et al.*, 1989). Close examination of their results show that 17ß-oestradiol dose-dependently reduces the spike phase of LH release, but the decrease in plateau phase secretion is only seen up to 1 nM 17ß-oestradiol and thereafter (at 10 and 100 nM) there is a tendency for the plateau of LH release to *increase* back towards control levels (King *et al.*, 1989). This means that high concentrations of 17ß-oestradiol disproportionately affect the two phases of LH secretion from gonadotroph cells, thereby altering the shape of the LH release profile.

It is possible that the concentration of plasma 17ß-oestradiol influences the GnRH-I-stimulated profile of LH secretion from the pituitary gland. The phasic components of LH secretion involve different intracellular signalling mechanisms used by GnRH-I (see SECTION 1.6). Gonadal steroids are known to affect different levels of the intracellular signalling pathway for example protein kinase C and the movement of calcium ions (see SECTION 1.6.4). Oestrogen could therefore modify the pattern of LH secretion from the pituitary gland of the adult chicken by affecting the process of stimulus-secretion coupling.

5.4 SUMMARY

The objective of this study was to test whether the high concentration of plasma 17ß-oestradiol in laying hens contributes to the sexually differentiated features of the adult LH responses to GnRH-I. This high concentration of 17ß-oestradiol was simulated in adult intact cockerels with an acute dose of oestradiol benzoate. The lack of effect of oestradiol benzoate on the duration of the GnRH-I-stimulated LH response indicates that there may be extrapituitary factors which contribute to this aspect of the sex difference, apart from the concentration of plasma 17ß-oestradiol. The results also show that the baseline concentration of plasma LH and the GnRH-I-stimulated release of LH *in vivo* were reduced by 17ß-oestradiol. The reduction could be mediated at the level of the pituitary gland and the hypothalamus. However in contrast to studies in sexually immature cockerels, these effects were not associated with changes in the contents of GnRH-I or GnRH-II in the hypothalamus of adult cockerels. This may indicate that measurements from large regions of the brain are insensitive to small changes in discrete brain areas. Alternatively, the differential effectiveness of 17ß-oestradiol to inhibit pituitary glands from adult cockerels and juvenile chickens *in vitro* could either suggest a maturation-related difference in the susceptibility of the pituitary gland to the actions of 17ß-oestradiol, or an indirect action of 17ß-oestradiol on the pituitary gland mediated by a change in hypothalamic function. A shift in the relative proportion of the concentrations of spike and plateau phase LH was induced by 17ß-oestradiol in GnRH-I-stimulated pituitary tissue from adult cockerels. It is concluded that elevating the concentration of circulating 17ß-oestradiol in the adult cockerel can reproduce some of the sexually differentiated characteristics of the laying hen.

6 THE DIRECT EFFECT OF GONADAL STEROIDS ON GONADOTROPH FUNCTION *IN VITRO*

6.1 INTRODUCTION

It is difficult to interpret observations from studies on the effects of gonadal steroids in the whole animal because of interactions between the components of the hypothalamic-pituitary-gonadal axis. Thus steroids may act on the hypothalamus to suppress the release of GnRH-I (SECTION 1.3) to reduce the stimulatory input to the pituitary gland and decrease the synthesis and release of LH from the gonadotroph cells. However this reduction in gonadotroph function could also result from the direct action of steroids on the gonadotroph cells themselves. To distinguish between the direct and indirect effects of steroids on gonadotroph cells, pituitary fragments were treated with 17ß-oestradiol *in vitro* (SECTION 5.2.3). However interpretation of these experiments is complicated by a number of problems; for example development of necrosis at the core of the tissue (Porter & Licht, 1985), slow passage of hormones, nutrients and waste material into and out of the tissue, and individual tissue variability (Evans *et al.*, 1984). These problems can be overcome by dispersing pituitary glands into single cells (Evans *et al.*, 1984). A method for preparing and culturing chicken pituitary cells *in vitro* was therefore developed paying particular attention to maintaining gonadotroph responsiveness to GnRH-I because the responsiveness of pituitary cells from rats has been shown to be sensitive to the method for cell culture (Wilfinger *et al.*, 1979). This chapter describes how exposure of pituitary cells from juvenile chickens to 17ß-oestradiol modifies the function of gonadotroph cells.

The laying hen is characterised by high concentrations of circulating 17ß-oestradiol with low concentrations of plasma testosterone, whereas the adult cockerel has a high concentration of plasma testosterone but low concentrations of plasma 17ß-oestradiol (Tanabe *et al.*, 1979, 1981; Knight, 1983; Stansfield & Cunningham, 1988). Testosterone may exert an inhibitory feedback action on LH release (Knight *et al.*, 1983; Wilson *et al.*, 1990b) through a reduction in the release of GnRH-I (Knight *et al.*, 1983; Lal *et al.*, 1990; Sharp *et al.*, 1990; Wilson *et al.*, 1990b) thereby reducing the frequency of episodic LH release (Wilson & Sharp, 1975c). However, there is also evidence for a direct effect of testosterone on the release of LH from pituitary gland of birds resulting in an enhancement or suppression of GnRH-stimulated release of LH *in vitro* (SECTION 1.5.2). In view of the uncertainty of the sites of action of testosterone on the hypothalamic-gonadotroph axis, experiments were carried out to investigate the direct action of testosterone on cultured pituitary cells from juvenile chickens.

Similar concentrations of plasma progesterone were found in the adult cockerel and laying hen (CHAPTER 3), however the possibility that progesterone may exert a direct effect on the release of LH from the pituitary gland has not been fully investigated (SECTION 1.5.2). A study was therefore carried out to establish the effect of progesterone on gonadotroph function *in vitro*.

6.2 RESULTS

6.2.1 Validation of gonadotroph cell function

6.2.1.1 Time-course of GnRH-I-induced LH release from cultured pituitary cells from juvenile chickens

GnRH-I stimulated two phases of LH accumulation from static cultures of pituitary cells (FIGURE 6.1). During the first 30-minutes of stimulation, LH secretion increased more than in control cultures not exposed to GnRH-I ($P<0.001$; FIGURE 6.1a). A peak of LH secretion was observed 10-minutes after GnRH-I stimulation, followed by a period when LH was released at a lower rate (60 - 180-minutes; $P<0.01$ compared with the peak rate of LH secretion; FIGURE 6.1b). A 60-minute time-point was chosen for use in later experiments because it corresponded to the terminal portion of the first phase of GnRH-I-induced LH release.

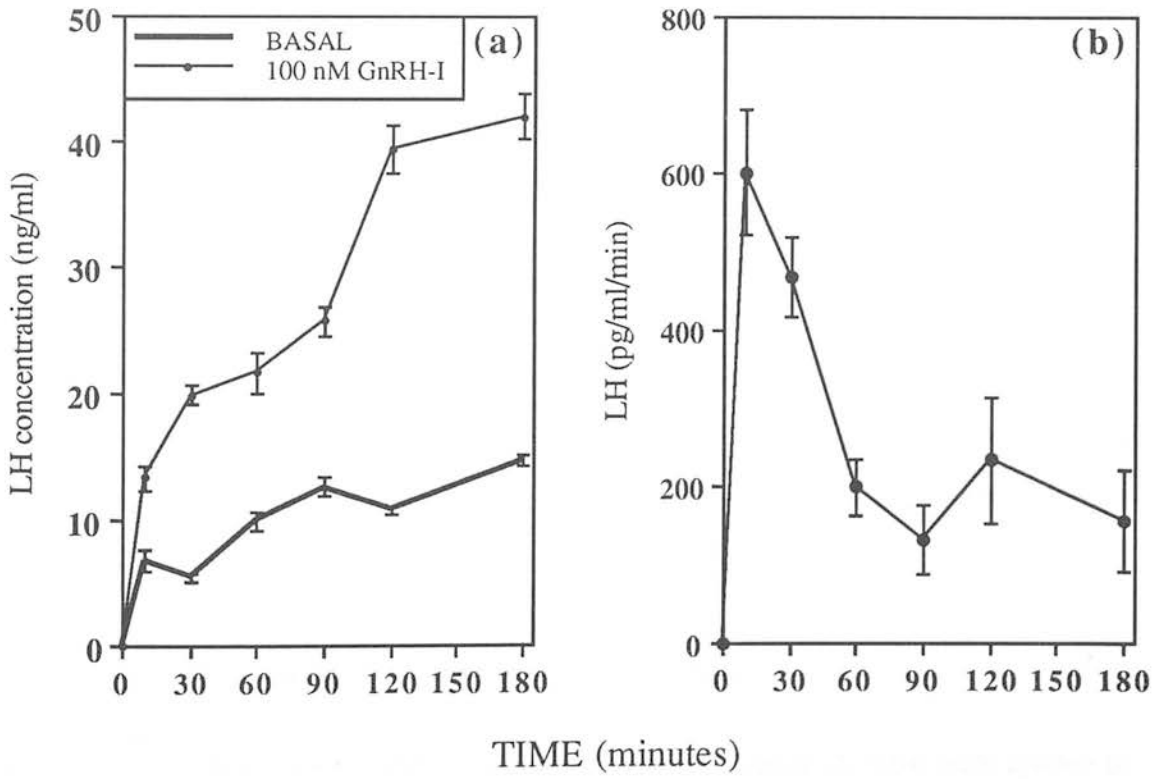


FIGURE 6.1: Time-course of LH secretion from cultured pituitary cells from juvenile chickens in response to GnRH-I.
Pituitary cells from juvenile chickens (500,000 cells/500 µl/well) cultured previously for 48-h were incubated for 180-min with or without 100 nM GnRH-I following the protocol described in SECTION 2.6.1 (n = 8 wells per treatment group per time-point). Curves start at zero where no LH release was assumed to have occurred. An analysis is presented of (a) the accumulated concentration of LH and (b) the rate of LH release (basal release subtracted; pg/ml/min).

6.2.1.2 Relationship between dose of GnRH-I and LH release

GnRH-I stimulated a concentration-dependent increase in LH secretion from 48-hour cultures of pituitary cells from juvenile chickens dispersed using collagenase or trypsin (FIGURE 6.2). The concentration of GnRH-I required to produce half-maximum release of LH (ED_{50}) was 1.7 nM and 1.8 nM for cells dispersed with collagenase or trypsin respectively. Cells dispersed using trypsin released more LH than cells dispersed using collagenase ($P<0.001$).

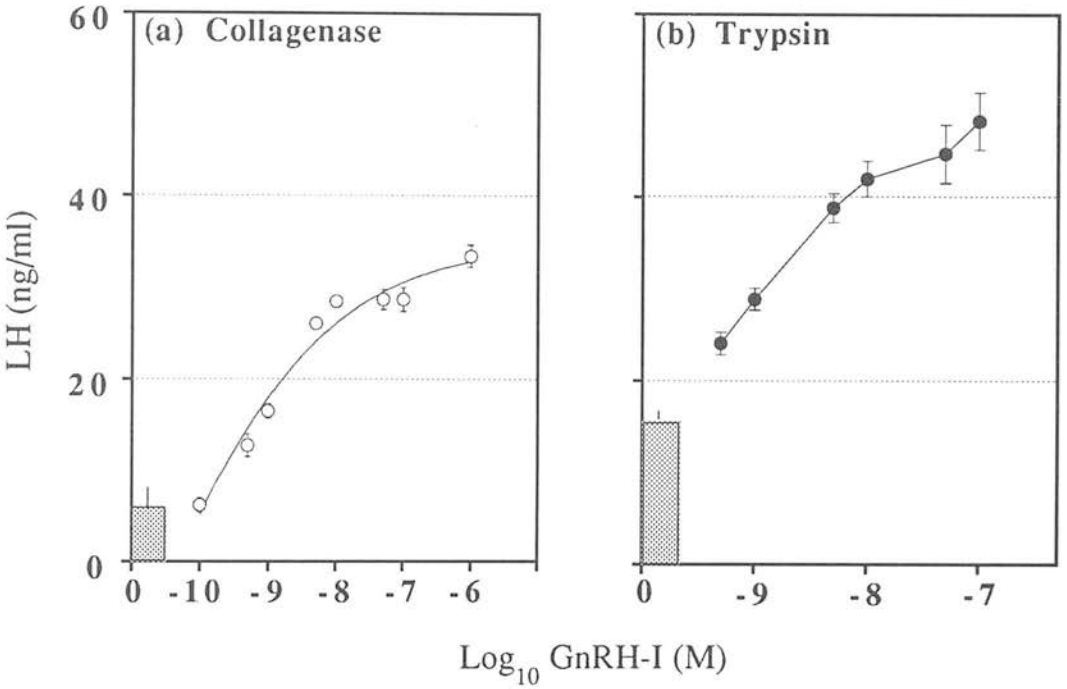


FIGURE 6.2: GnRH-I dose-response curves for cultured pituitary cells from juvenile chickens.
Pituitary glands from juvenile chickens were dispersed with (a) collagenase or (b) trypsin and cultured for 48-h (500,000 cells/500 µl/well). After rinsing in fresh medium, the cells were incubated with increasing doses of GnRH-I for 60-min (n = 8 per dose). The shaded bars show the release of LH in the absence of GnRH-I.

6.2.2 Cultures of pituitary cells from adult chickens

When the conditions used for culturing pituitary cells from juvenile chickens were applied to collagenase-dispersed pituitary cells from adult cockerels and hens, only those from cockerels released LH in response to GnRH-I (in 5 out of 8 experiments; $P<0.05$ at all GnRH-I doses compared with control). The maximum increase in LH secretion was 1.6-fold of basal LH release after stimulation with 1 nM or more GnRH-I (FIGURE 6.3); no increase in LH secretion was detected with less than 1 nM GnRH-I. No increase in LH release was detected from cultured pituitary cells from laying hens after the addition of GnRH-I in 8 separate experiments (FIGURE 6.3).

Pituitary cells from adult chickens were incubated with the protein kinase activator 12-O-tetradecanoyl phorbol acetate (TPA) in order to establish whether the poor LH responses were due to a failure of the signal transduction pathway for LH release. Pituitary cells from adult cockerels and laying hens released LH in response to TPA (FIGURE 6.3) with similar efficacies and potencies of 5.3-times basal and 20 nM in the male, and 5.6-times basal and 25 nM in the female respectively.

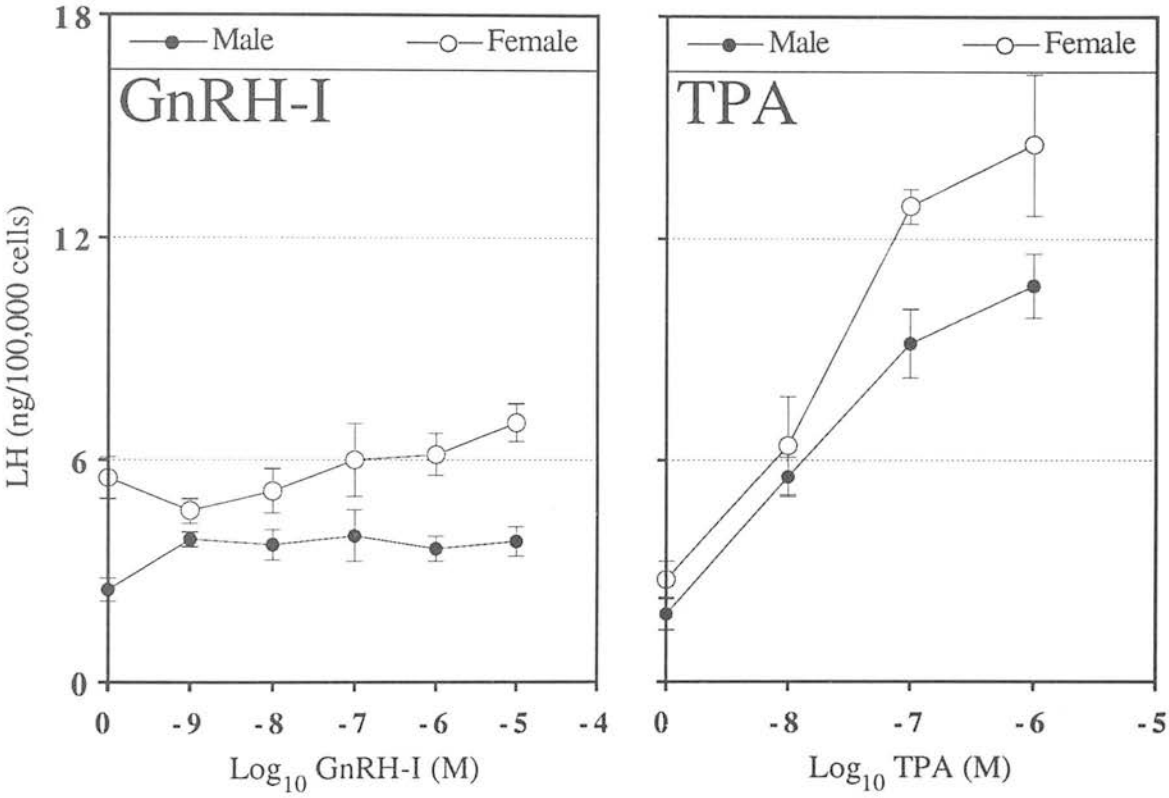


FIGURE 6.3: Dose-response curves for GnRH-I and TPA-induced LH release from 48-hour cultures of pituitary cells prepared from adult cockerels and hens.
Pituitary cells (48-h cultures; 100,000 cells/200 µl/well) from adult males or females were incubated for 60-min with various concentrations of GnRH-I or TPA (n = 6 per treatment group).

In view of the poor response of cultured pituitary cells from adult chickens to GnRH-I, an evaluation was made of the conditions for the dispersal and culture of pituitary cells from adult cockerels (FIGURE 6.4). The optimum conditions were found to be the same as for the pituitary cells from juvenile chickens over a period of 4 - 96-hours in culture. Pituitary cells from adult cockerels prepared using trypsin released more basal and GnRH-I-stimulated LH ($P<0.05$ for each treatment) than those prepared with collagenase (FIGURE 6.4), irrespective of the serum concentration used (no significant difference between 5% and 10% FCS). Cells cultured with DMEM released either little or no LH in response to 100 nM GnRH-I ($\Delta LH_{collagenase} = 3.5 \pm 0.6$, $\Delta LH_{trypsin} = 1.3 \pm 0.5$ ng/ml), whereas those maintained in M199 always released more LH ($\Delta LH_{collagenase} = 5.9 \pm 0.5$, $\Delta LH_{trypsin} = 6.3 \pm 1.0$ ng/ml; both $P<0.01$ compared with the respective

LH responses of DMEM-cultured cells). The amounts of LH released by a depolarising concentration of high K^+ (K^+-releaseable LH) from cultures of pituitary cells from adult cockerels after 24, 48, 72 and 96-hours in culture were 9.0 ± 1.1, 12.5 ± 2.6, 8.2 ± 1.7 and 6.3 ± 1.7 ng/100,000 cells/30-minutes respectively (basal LH values subtracted; n = 6).

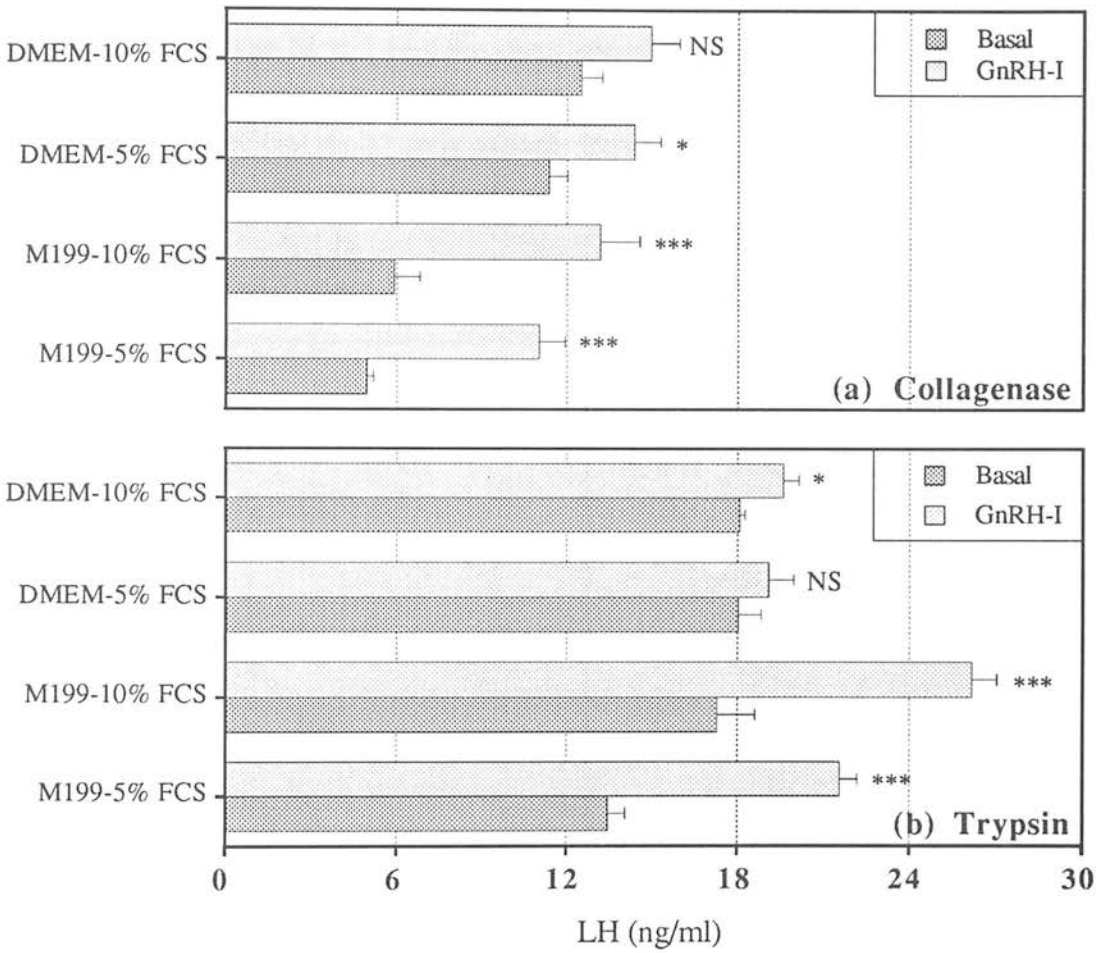


FIGURE 6.4: Evaluation of the dispersion and culture conditions for pituitary cells from adult cockerels.
Pituitary glands from adult cockerels were dispersed by (a) collagenase or (b) trypsin, and the cells (100,000 cells/200 µl) cultured for 48-h in 5 or 10% charcoal-stripped FCS, in bicarbonate-buffered M199 or DMEM. Cells were incubated for 60-min with or without 100 nM GnRH-I. NS = not significantly different, *P<0.05 and ***P<0.001 compared with the respective basal LH (n = 6).

6.2.3 Effect of pretreatment with 17ß-oestradiol, testosterone or progesterone on gonadotroph function

6.2.3.1 Effect of steroids on basal LH release

Treatment of cultures of pituitary cells from juvenile chickens with 17ß-oestradiol, testosterone or progesterone (0 - 1 µM) for 24 or 48-hours did not affect the baseline release of LH. However, treatment with 10 µM all of these steroids for 48-hours significantly increased (P<0.05, n = 8) the basal release of LH over controls.

6.2.3.2 Effect of dose of steroid on GnRH-I-stimulated release of LH

Similar reductions of GnRH-I-induced release of LH were seen after treating pituitary cells from juvenile chickens with testosterone, progesterone or 17ß-oestradiol. The maximum inhibitions for testosterone, progesterone or 17ß-oestradiol were respectively 48 ± 2, 51 ± 3 and 50 ± 2% of the control LH response ($P<0.001$; determined from raw data; FIGURE 6.5). By comparing the steroid concentration which inhibited the control LH response by 50% of the maximum response (IC_{50}), 17ß-oestradiol was 18 and 48-times more potent than testosterone and progesterone respectively. A high concentration of 1000 nM 17α-oestradiol reduced the GnRH-I-stimulated release of LH by 12 ± 4% (not significantly different from control) but in terms of the IC_{50} value, 17ß-oestradiol was 3063-times more potent than 17α-oestradiol.

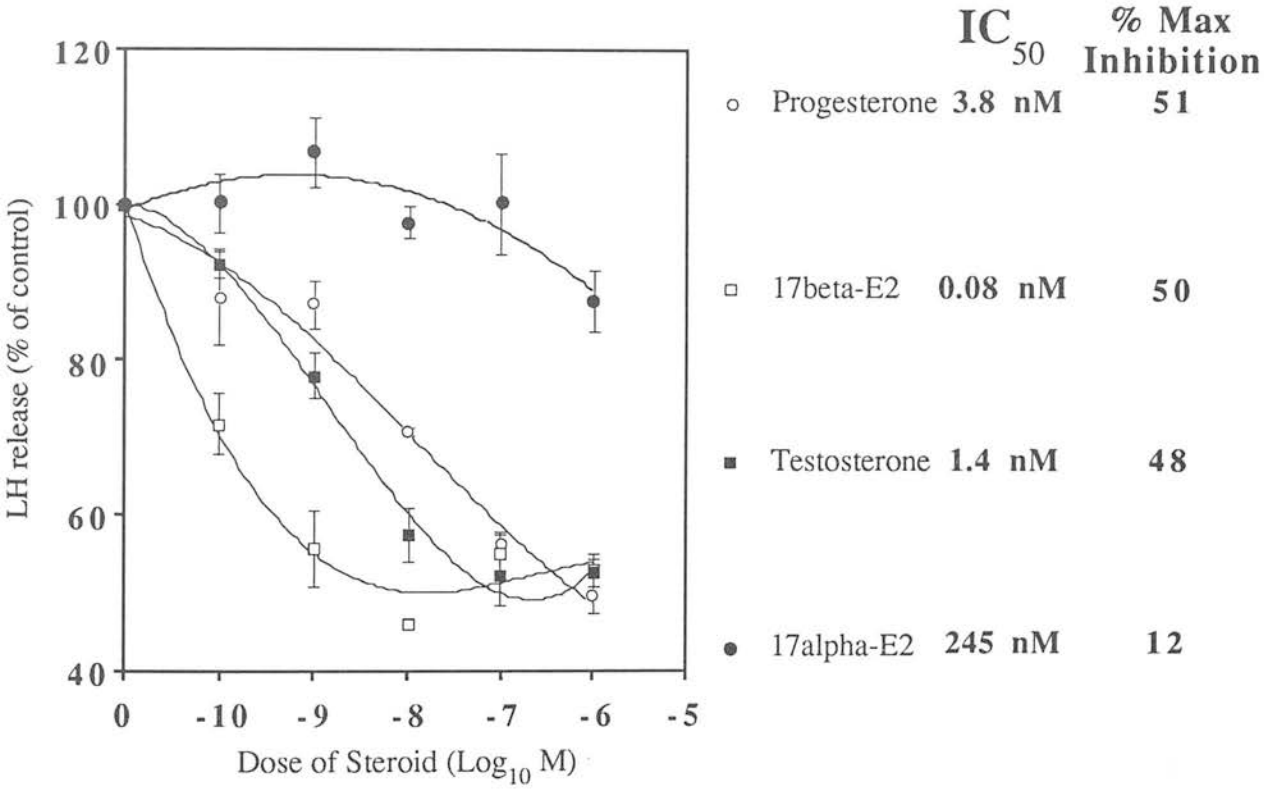


FIGURE 6.5: Relative ability of steroids to suppress the pituitary LH response to GnRH-I.
Anterior pituitary cells from juvenile chickens (200,000 cells/200 µl) were cultured with steroids (E2 = oestradiol) for 48-h before incubating with 100 nM GnRH-I for 60-min. Basal LH was subtracted and the results expressed as a percentage of control values (n = 6). IC_{50} represents the concentration of steroid required to produce half-maximum inhibition of LH release; note - for progesterone and 17α-oestradiol IC_{50} values, half-*maximal* concentrations were calculated because a *maximum* response was not reached. Similar, though less pronounced changes were seen in cultures treated with steroids for 24-h (not shown).

6.2.3.3 Effect of steroids on the GnRH-I dose-response curve

Treatment of pituitary cells with 1 nM testosterone, progesterone or 17ß-oestradiol equally reduced the maximum release of LH induced by 10 µM GnRH-I by 15, 17 and 18% respectively ($P<0.05$

determined from raw data; FIGURE 6.6). However, the LH responses of cells treated with 17α-oestradiol were not reduced. None of the steroids altered the ED_{50} value for GnRH-I.

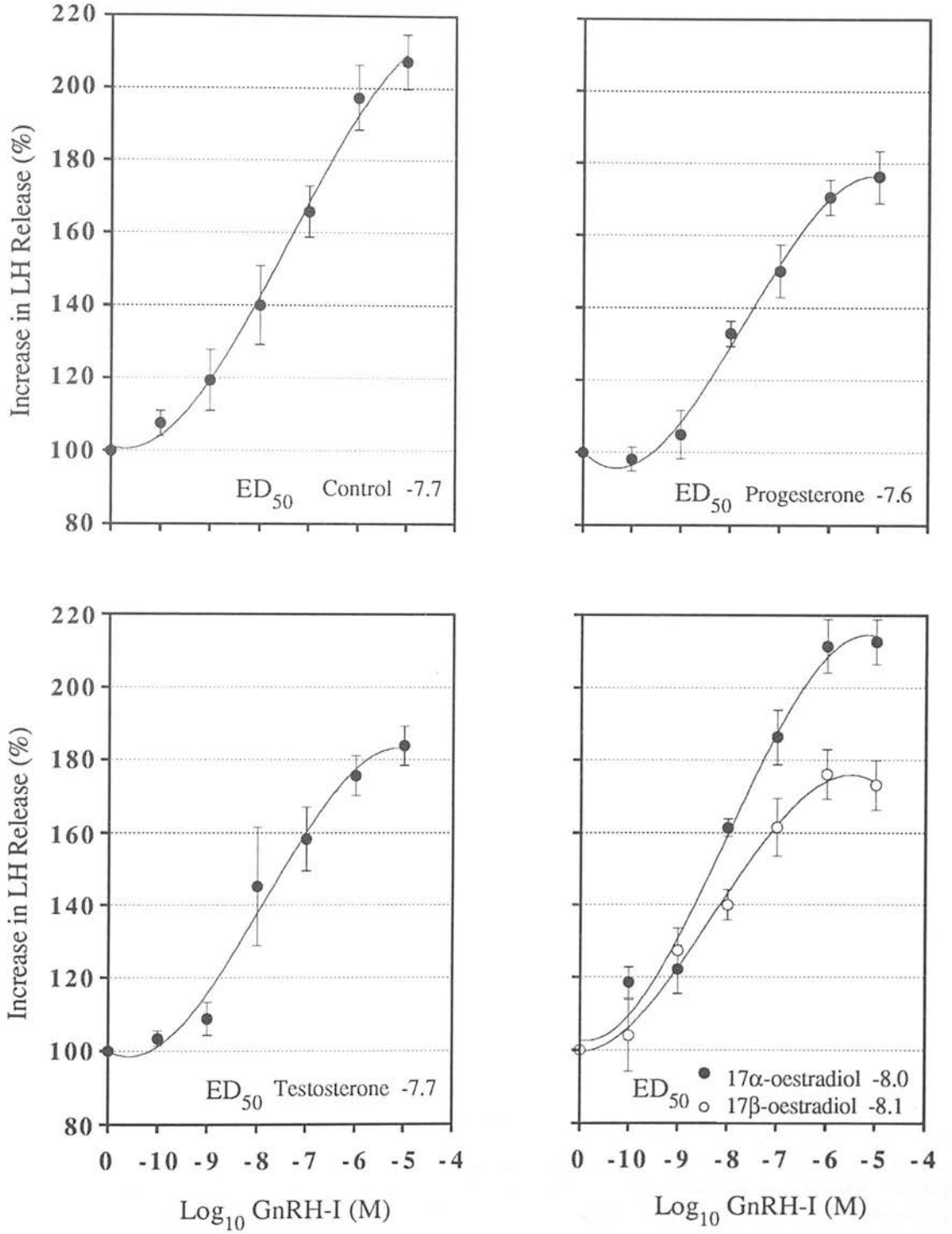


FIGURE 6.6: Effect of steroid treatment on the sensitivity and responsiveness of pituitary cells to GnRH-I.
Pituitary cells from juvenile chickens (200,000 cells/200 µl) were cultured in (a) the absence or presence of (b) 1 nM progesterone, (c) 1 nM testosterone, or (d) 1 nM 17α-oestradiol or 17ß-oestradiol for 48-h before incubating with 0 - 10 µM GnRH-I for 60-min. Results are expressed as a percentage of basal LH release (n = 6). The ED_{50} (expressed as Log_{10} M concentration) shown on the graphs represent the concentration of GnRH-I required to produce half-maximum stimulation of LH release. Similar, though less pronounced changes were found after 24-h of treatment with steroids (not shown).

6.2.3.4 Effect of steroids on the K^+-releaseable and total cellular LH

Treatment of pituitary cells from juvenile chickens with testosterone, progesterone or 17ß-oestradiol for 48-hours significantly reduced the K^+-releaseable amount of LH ($P<0.01$ at 1 µM steroid compared with no steroid; determined from raw data; FIGURE 6.7) and the total pituitary content of LH ($P<0.01$; FIGURE 6.8). Testosterone, progesterone and 17ß-oestradiol were equally effective in reducing the total pituitary LH content, but the maximum inhibition of the K^+-releaseable store of LH by testosterone was less than that for 17ß-oestradiol or progesterone ($P<0.05$). The stereoisomer 17α-oestradiol did not significantly affect the K^+-releaseable store or total cellular content of LH (determined from the raw data).

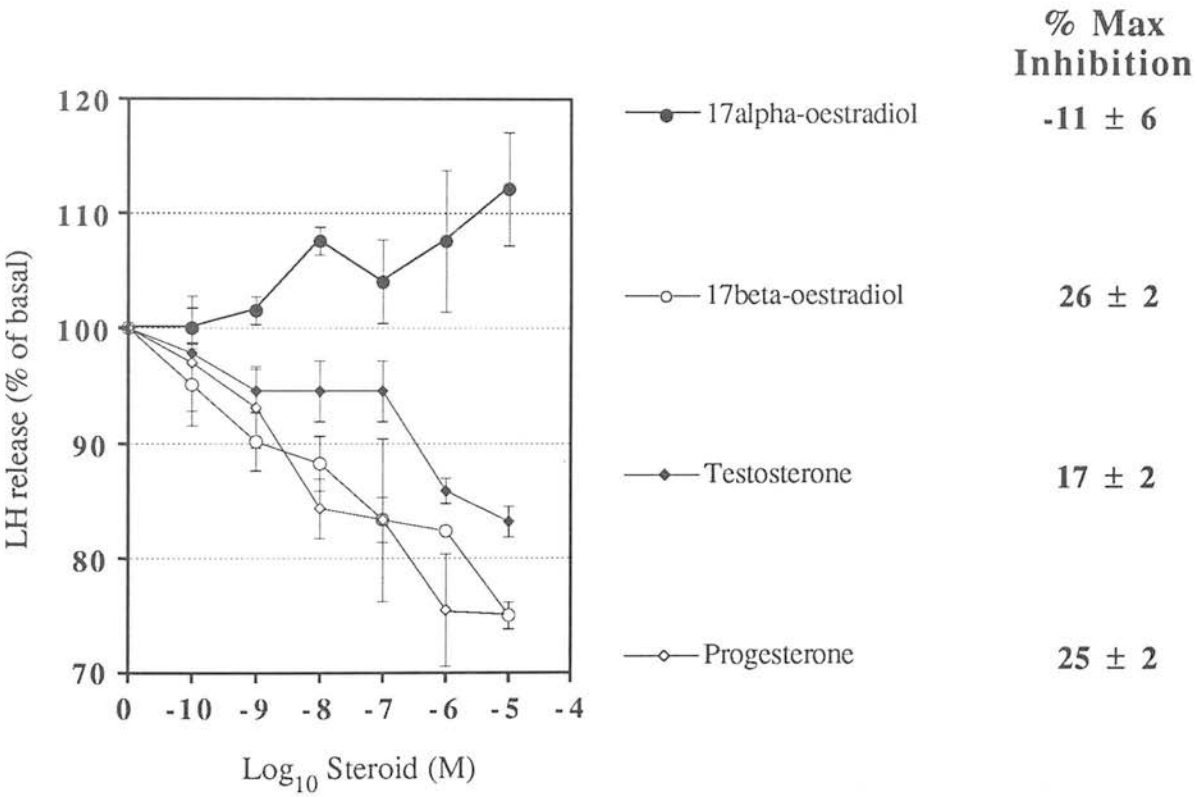


FIGURE 6.7: Effect of steroid treatment on the release of LH by 60 mM K^+.
Pituitary cells from juvenile chickens (200,000 cells/200 µl) were treated for 48-h with steroid and then depolarised with 60 mM K^+ in Buffer A-NBCS for 30-min. Results are expressed as a percentage of values observed in the absence of steroid (control); n = 6. The maximum inhibitory effect by each steroid (10 µM) on LH release is expressed as a percentage of the control response. Similar, though less pronounced changes were seen after 24-h treatment with steroids (results not shown).

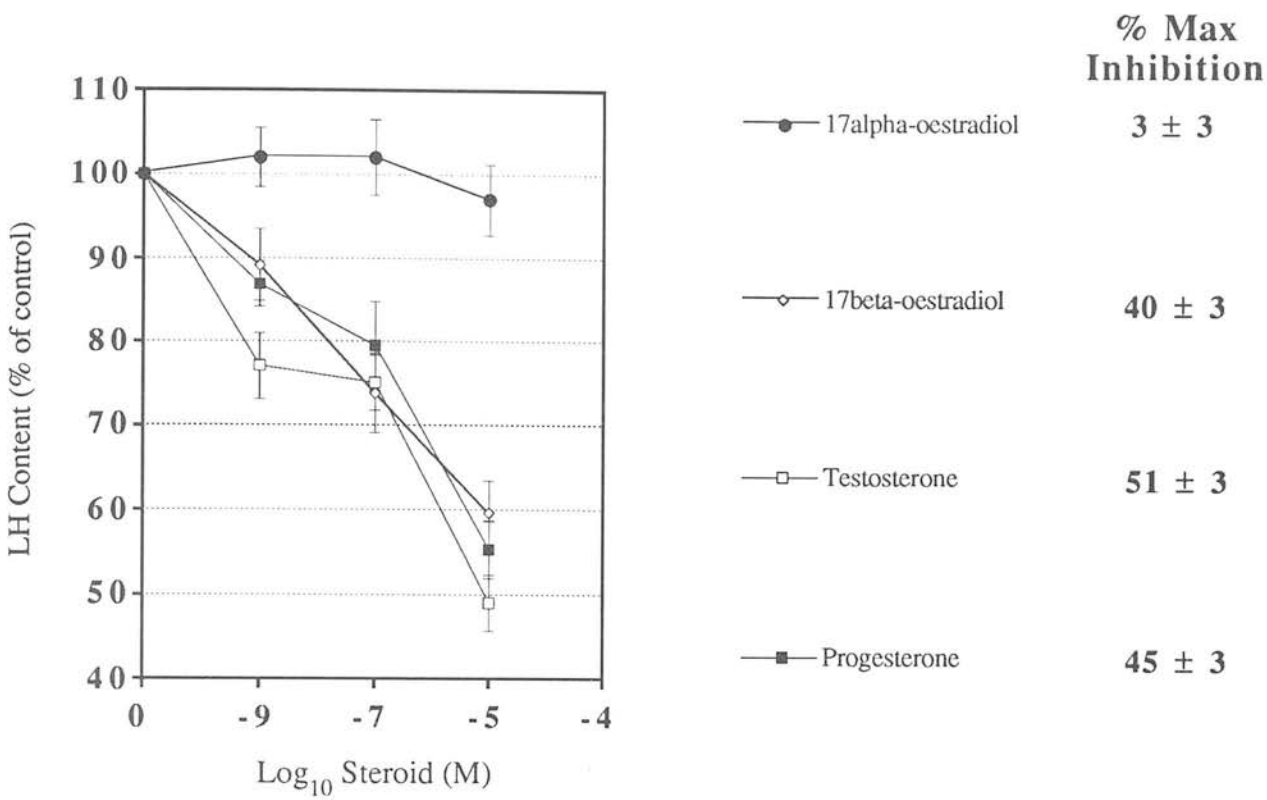


FIGURE 6.8: Effect of steroid treatment on the total pituitary cell content of LH. See FIGURE 6.7 for legend. Instead of stimulating the cells with GnRH-I, the cultures were lysed with 1% (v/v) Triton-X100. A similar though less pronounced depression in LH content was seen in pituitary cells treated with steroids for 24-h (results not shown).

6.3 DISCUSSION

The observations presented in CHAPTER 5 show that 17ß-oestradiol suppresses LH release in adult cockerels, either by a direct action on the pituitary gland or indirectly, by decreasing the release of GnRH-I. In order to determine whether 17ß-oestradiol acts directly on the pituitary gland, a method for dispersing and maintaining chicken pituitary cells was established. Pituitary cells were prepared from 5 - 7-week-old juvenile chickens of mixed sexes, which have low concentrations of plasma gonadal steroids compared with adult birds (Tanabe *et al.*, 1979, 1981). Cells from juvenile chickens are therefore suitable to investigate the direct effects of 17ß-oestradiol *in vitro* on LH synthesis and release. The effects of testosterone and progesterone on gonadotroph function were also investigated to establish their potency relative to that of 17ß–oestradiol.

6.3.1 Conditions for pituitary cell culture

6.3.1.1 Dispersion

Animal cells are organised into tissues and held together by a complex matrix of different large molecular weight substances including proteins, glycoproteins, lipids, glycolipids and mucopolysaccharides (Freshney, 1987). Ideally, tissues should be dispersed into individual cells

by selectively degrading these intercellular molecules without affecting the functional integrity of the cell membrane, for example hormone binding sites. In practice however, the absolute preservation of the function of cell membranes is not always possible. Collagenase and trypsin-based methods were compared for their suitability in the present experiments.

The collagenase method for isolating chicken pituitary cells was chosen for studies on the regulation of LH release because of the lower baseline release of LH from collagenase-dispersed cells and the larger release in response to GnRH-I, compared with the trypsin-dispersed cells. However, whereas trypsin generated a suspension of almost entirely single cells, small undissociated clusters of 2 - 4 cells remained when collagenase was used. Trypsin-prepared cells were therefore used in techniques where a suspension of single cells was essential (i.e. membrane fluidity and experiments; see CHAPTER 7). Both fresh and cultured trypsin-dispersed gonadotroph cells responded to GnRH-I with an increased release of LH although the cells were also associated with a high basal release of LH. It is possible that this high background level of LH was due to leakage of LH from damaged cells.

The difference between trypsin and collagenase-dispersed cells on the LH responsiveness to GnRH-I may relate to the mechanism of action of each enzyme. Trypsin acts on the peptide bonds on the carboxyl side of lysine and arginine residues for its dissociating properties (Stryer, 1981). The crude collagenase preparation used in these experiments contains collagenase, caseinase, clostripain and trypsin activity which is a gentler combination of enzymes for dissociating cells than using trypsin alone (Freshney, 1987). However, collagenase removes sialic acid from cell membrane-associated glycolipids (Rosenthal & Fain, 1971). These sialic acid-containing gangliosides are important in binding cations and are thought to have pronounced effects on the ionic permeability of cell membranes (Bowman & Rand, 1982).

Freshly prepared cells may lose their responsiveness to GnRH stimulation because of a decrease in GnRH receptor number or affinity for GnRH (Hopkins, 1977; Zilberstein *et al.*, 1983), or to uncoupling of a post-receptor mechanism (Smith *et al.*, 1983; Gorospe & Conn, 1987a, 1987b, 1988; Chang *et al.*, 1988a). This effect is more pronounced with trypsin than collagenase (Hopkins, 1977) and cells dispersed by collagenase also recover more quickly after a period in culture (Hopkins, 1977). The present study showed that pituitary cells from juvenile chickens responded to GnRH-I immediately after dissociation with either trypsin or collagenase, and for up to 72-hours in culture. Similar ED_{50} values for GnRH-I were calculated for 48-hour cultures of trypsin and collagenase-dispersed cells which shows that, irrespective of the dissociation agent used, the mechanisms by which GnRH-I stimulates gonadotroph cells are similar and remain intact. However, others find that chicken pituitary cells dispersed by trypsin, lose their responsiveness to GnRH when placed into culture (Bonney & Cunningham, 1977a).

As reported previously (Johnson *et al.*, 1984), different batches of pituitary cells produced quantitatively differing LH responses to GnRH stimulation, but reproducible trends were always

seen. These differences may result from slight day-to-day variations in the procedures for collecting and dispersing the pituitary glands (Johnson *et al.*, 1984).

6.3.1.2 Effect of serum

This study showed that the serum concentration for sustaining gonadotroph function was not as important as the species from which the serum originated (TABLE 2.4). A supplement of 5 - 20% serum is usually added to culture media to provide undetermined factors which support cell attachment and growth (Freshney, 1987). Foetal calf serum (FCS) is the most widely used source of these factors and performed better than chicken serum in maintaining the function of gonadotroph cells *in vitro*.. Chicken serum did not support chicken gonadotroph cells as well as FCS which suggests that FCS contains a richer supply of factors than serum from juvenile chickens. Another study which optimised the culture conditions for somatotroph cells of the juvenile chicken, found that neither the concentration or type of serum affected the growth hormone content of 4-day cultures (Vasilatos-Younken, 1986). Foetal calf serum was therefore used in preference to chicken serum because of its better performance in sustaining gonadotroph function, and to avoid the potential problem of cross-reaction of LH in chicken serum in the chicken LH radioimmunoassay.

All sera were stripped using charcoal-dextran to remove steroids which might interfere with the experiments (SECTION 2.6.1). This procedure reduced the GnRH-I-stimulated release of LH from gonadotroph cells in a parallel fashion, and perhaps indicates removal of gonadotroph-promoting factors normally present in FCS. Since charcoal-stripping FCS does not affect the protein content of the serum (O'Conner *et al.*, 1988) the factors are possibly not proteins. However these factors may represent highly potent peptides present only in small quantities.

6.3.1.3 Effect of phenol red on gonadotroph function

Phenol red reduced the basal and GnRH-I-stimulated release of LH by 37% (FIGURE 2.9). This indicated that gonadotroph cells of juvenile chickens were sensitive to this oestrogenic chemical (SECTION 2.6.4.2.2) and also that these cells respond to oestrogens. This is an important observation because previous studies on cultured chicken pituitary cells include phenol red in the culture medium (King *et al.*, 1986; Davidson *et al.*, 1988; King *et al.*, 1989; Johnson & Tilly, 1991). Phenol red-free medium was used in all subsequent experiments to avoid the oestrogenic effects of phenol red.

6.3.1.4 Effect of culture medium and buffer on gonadotroph function

Dulbecco's modified Eagle's medium (DMEM) and Medium 199 (M199) were compared on the basis of how well they could sustain gonadotroph function in culture. In this respect, M199 was better than DMEM and may be due to the richer content and greater range of amino acids and vitamins in M199 (Freshney, 1987). When comparing the effect of two buffers on gonadotroph

function, media buffered with bicarbonate appeared to perform better than media buffered with HEPES. This was noted even in freshly dispersed cells and was associated with a greater basal release of LH. HEPES has been used previously with chicken pituitary cells (Chou *et al.*, 1985; King *et al.*, 1986; Johnson & Tilly, 1991). However the present results indicate that bicarbonate-buffered media maintains the function of cultured gonadotroph cells better than HEPES. An explanation for this difference is not available.

The best medium for the culture of pituitary cells from juvenile chickens was found to be phenol red-free M199 buffered with bicarbonate and supplemented with FCS. Other formulations for culturing these cells consist of M199-HEPES-BSA (Chou *et al.*, 1985), DMEM-HEPES-FCS (King *et al.*, 1986), M199-HEPES with no serum (Johnson & Tilly, 1991), or bicarbonate-buffered M199 supplemented with either horse serum and chicken embryo extract (Hasegawa *et al.*, 1984), or FCS (Perez *et al.*, 1989). Finally, by comparing the effect of five types of culture medium on the highest chicken growth hormone content, the media were ranked αMEM > RPMI-1640 > EBSS > DMEM > M199 (Vasilatos-Younken, 1986; no information was given on the buffer system used). It is possible therefore that αMEM, RPMI-1640 and EBSS media might perform even better than M199 or DMEM with respect to supporting chicken gonadotroph cells in culture.

6.3.1.5 Validation of gonadotroph function in culture

6.3.1.5.1 Pituitary cells from juvenile chickens

In common with the perifused pituitary fragments (CHAPTER 4), GnRH-I stimulated a biphasic accumulation of LH from static incubations of pituitary cell cultures. This indicates that chicken gonadotroph cells retain their functional capacity to secrete LH in response to GnRH-I after the dissociation and culture procedures. This phasic pattern of LH accumulation is similar to the profile of LH release from chicken pituitary cells described by Smith *et al.* (1987) and Davidson *et al.* (1988).

6.3.1.5.2 Pituitary cells from adult chickens

Adult pituitary cells were poorly responsive to GnRH-I stimulation (FIGURE 6.3) compared with cultures of pituitary cells from juvenile chickens (a maximum of 4.5 - 6-fold of basal LH; FIGURE 6.2). Pituitary cells from juvenile but not sexually mature male turkeys also show this maturational difference in responsiveness to GnRH (Godden *et al.*, 1977). A low responsiveness to GnRH stimulation has been reported previously for pituitary cells from adult cockerels (1.8-fold of basal LH, Johnson *et al.*, 1984) and laying hens (1.4-fold of basal LH, Kawashima *et al.*, 1982; 1.2 - 2.3-fold of basal LH, Wilson *et al.*, 1990a). The reason for these observations is unlikely to be due to deprivation of GnRH-I when the pituitary cells from adult chickens are placed into culture because those from juvenile chickens responded well to GnRH-I stimulation under identical conditions. It is also unlikely that the cells from adults were stimulated for an insufficient period of time, because LH is released within 2-minutes of GnRH-I administration *in vivo* and *in*

vitro (FIGURE 3.1 and 4.9). The maturational difference in maximum responsiveness to GnRH of cultures of pituitary cells from juvenile and adult chickens is not seen in rats (Tang, 1978). Since non-dissociated pituitary tissue from adult chickens secreted large amounts of LH in the presence of GnRH-I (FIGURE 4.9) but freshly dispersed cells did not (SECTION 6.2.2), the function of gonadotroph cells may be compromised by the dispersion procedure. The dispersion procedure may disturb the stimulus-secretion coupling pathway at a level preceding protein kinase C activation i.e. between the GnRH-receptor and formation of the endogenous protein kinase C activator, 1,2-diacylglycerol, because these same adult pituitary cells were responsive to the protein kinase C activator 12-O-tetradecanoyl phorbol acetate (FIGURE 6.3). However, it is not understood why gonadotroph cells from adult but not juvenile chickens should be sensitive to the dispersion procedure.

Pituitary cells form intercellular communications with each other (Denef *et al.*, 1989), or establish close paracrine control mechanisms (see SECTION 1.5.3), and these could be damaged or destroyed by the dispersion procedure, and pituitary cells from adult chickens could be more sensitive than those from juveniles. One possibility is suggested by the inhibitory effect of elevated concentrations of plasma prolactin on LH secretion in broody bantam hens (Sharp *et al.*, 1988). In mammals, prolactin has been suggested to reduce the number of GnRH-receptors and the basal release of gonadotrophins from the pituitary gland of the rat (Marchetti & Labrie, 1982). In the chicken, the release of prolactin from pituitary tissue increases in the presence of 17ß-oestradiol (Hall *et al.*, 1984), and concentrations of plasma prolactin are higher in adult than in juvenile cockerels (Sterling *et al.*, 1984b) and hens (PJ Sharp, personal communication). It is therefore possible that an accumulation of prolactin in static cultures of pituitary cells from adult chickens may directly suppress the function of gonadotroph cells.

6.3.2 Steroids and gonadotroph function

The present study using a cell culture system showed that 17ß-oestradiol acts directly on the pituitary gland of sexually immature chickens to suppress gonadotroph function. This was evident after 24 to 48-hours of treatment and characterised by a reduction in the GnRH-I and K^+-induced release of LH, and a decrease in the total cellular content of LH. The direct inhibitory effect of 17ß-oestradiol on the LH response to GnRH-I is consistent with previous studies on the chicken (Luck & Scanes, 1980; King *et al.*, 1989) but differs from the increased LH responses of turkey pituitary cells in the presence of 17ß-oestradiol (Knapp *et al.*, 1987). One report indicates that the effect of 17ß-oestradiol on LH secretion from chicken pituitary cells depends on the duration of treatment; with short incubations (<2-hours) suppressing LH release, and longer incubations (4 - 18-hours) enhancing the response to GnRH (Bonney & Cunningham, 1977d). This short-term inhibitory effect of 17ß-oestradiol has been confirmed by Luck and Scanes (1980). However, King *et al.* (1989) found no effect of 17ß-oestradiol after 1 and 6-hours of treatment, on the LH response to GnRH-I but that at least 24-hours was required to significantly inhibit the response. These observations in chickens differ from the effect of 17ß-oestradiol on mammalian gonadotroph cells.

In mammals, 17ß-oestradiol exerts a time-dependent action on gonadotroph function, initially inhibiting and later enhancing the LH response to GnRH (reviewed by Brann & Mahesh, 1991). The suppressive action of 17ß-oestradiol on the magnitude of GnRH-I-stimulated LH release from cultures of pituitary cells from juvenile chickens contrasts with the absence of a suppressive effect of 17ß-oestradiol on GnRH-I-induced LH release from cultures of pituitary fragments from adult cockerels (CHAPTER 5). The difference between these observations could be due to a differential ability of 17ß-oestradiol to penetrate throughout the two tissue preparations.

The suppressive effect of 17ß-oestradiol *in vitro* and *in vivo* (CHAPTER 5) on the total content of LH from pituitary tissue from juvenile chickens is consistent with the reduction in pituitary content of LHß-mRNA in juvenile hens injected with oestradiol benzoate (Kallmeier *et al.*, 1991), and indicates a decrease in LHß-subunit synthesis (discussed in SECTION 5.3). Oestrogen-receptors are found in the chicken pituitary gland (Kawashima *et al.*, 1987) and confined almost exclusively to the gonadotroph cell population of pituitary glands from chick embryos (Gasc *et al.*, 1980). This would explain the stereospecific requirement of oestrogen action because 17α-oestradiol did not affect gonadotroph function. The presence of oestrogen-receptors in the cell nucleus (Kawashima *et al.*, 1987) also suggests an effect of 17ß-oestradiol on gene expression. A similar distribution of oestrogen-receptors has also been described in mammals (Lieberburg & McEwen, 1977; Thieulant *et al.*, 1984; Thieulant & Duval, 1985; Krey & Kamel, 1990a). This indicates that 17ß-oestradiol can act on gonadotroph cells of the juvenile chicken to suppress LH synthesis at the genomic level, independently of a depressive effect of 17ß-oestradiol on GnRH-I release from the hypothalamus.

A high concentration of plasma testosterone in adult cockerels exerts a negative feedback effect on plasma LH, rather than a high concentration of 17ß-oestradiol as in laying hens. Testosterone and 17ß-oestradiol exert similar inhibitory effects on the function of gonadotroph cells from juvenile chickens *in vitro*, although the inhibitory potency of testosterone on the GnRH-I-stimulated release of LH was less than that for 17ß-oestradiol. Since aromatase activity has been found in the anterior pituitary gland of birds (Sharp *et al.*, 1986a; Callard *et al.*, 1990; Schlinger & Arnold, 1991), this suggests that part of the suppressive activity of testosterone on gonadotroph function could be mediated through aromatisation to 17ß-oestradiol. In view of the reduction in plasma LH by injections of testosterone and the associated decrease in release of GnRH-I (Knight *et al.*, 1983; Wilson *et al.*, 1990b), both the pituitary gland and hypothalamus could be affected by testosterone through the formation of 17ß-oestradiol. The question therefore arises as to why the high concentration of plasma testosterone through its local conversion to 17ß-oestradiol by the hypothalamus and pituitary gland of adult cockerels, does not reproduce the inhibitory effects on LH release of high circulating levels of 17ß-oestradiol in laying hens. Four possibilities are considered.

First, the reported concentrations of plasma testosterone in adult cockerels represent the *total* concentration of extracted testosterone (Culbert *et al.*, 1977; Sharp *et al.*, 1977; Tanabe *et al.*,

1979; Knight, 1983) rather than the *free* and therefore bioavailable levels of testosterone. Circulating steroids associate with plasma proteins such as albumin or a specific sex hormone-binding protein (reviewed by Selby, 1990). A sex hormone-binding protein is present in the plasma of amphibia, reptiles and mammals (Ozon *et al.*, 1971; Corvol & Bardin, 1973; Martin & Ozon, 1975; Sernia, 1978; Selby, 1990). However, although there is a corticosteroid-binding protein in the chicken with some androgen-binding capacity and no oestrogen-binding capacity, there is no specific testosterone-binding protein (Wingfield *et al.*, 1984). It is therefore unlikely that a sex difference exists in the bioavailability of testosterone or 17ß-oestradiol in the adult chicken.

Second, testosterone may be inactivated by the high 5ß-reductase activity in the avian hypothalamus and pituitary gland to form 5ß-DHT (Bottoni & Massa, 1981; Massa & Sharp, 1981; Balthazart & Ottinger, 1984; Balthazart, 1991) which does not suppress the concentration of plasma LH in birds (Davies *et al.*, 1980; Massa & Sharp, 1981), thus rendering testosterone unavailable as a substrate for aromatase action.

Third, the conversion of testosterone to 17ß-oestradiol by aromatase activity in the pituitary gland and hypothalamus of the cockerel may be regulated such that the local levels of 17ß-oestradiol do not achieve the high concentrations found in the plasma of laying hens.

Fourthly, sexual maturation in the cockerel is associated with an apparent reduction in the sensitivity of the hypothalamic-pituitary complex to the inhibitory effect of testosterone on plasma LH (see SECTION 3.3.1.3). This could also be interpreted as a weaker suppressive effect of testosterone than 17ß-oestradiol on the concentration of plasma LH as found in cockerels (Massa & Sharp, 1985), and on the GnRH-I-stimulated release of LH from pituitary cells from juvenile chickens (King *et al.*, 1989; SECTION 6.2.3). This is the most likely explanation for why the high concentration of plasma testosterone in adult cockerels does not simulate the potent suppressive effect of high 17ß-oestradiol on plasma LH in laying hens.

Progesterone exerts a stimulatory effect on LH release in laying hens (Wilson & Sharp, 1976a), but not in prepubertal hens (Wilson & Sharp, 1975b). These *in vivo* effects of progesterone are thought to be mediated by releasing GnRH-I from the hypothalamus of the laying hen, rather than by a stimulatory action on the pituitary gland (Wilson & Sharp, 1975a, 1975b; Wilson *et al.*, 1990a). In fact, injection of progesterone into laying hens reduces the responsiveness of the pituitary cells to GnRH-I *in vitro* (Wilson *et al.*, 1990a), and progesterone also suppresses the GnRH-I-induced release of LH from pituitary cells from juvenile chickens (King *et al.*, 1989). This latter observation was confirmed in the present study. It is concluded that although the stimulatory effect of progesterone in laying hens is mediated through the release of GnRH-I from the hypothalamus, pituitary cells from juvenile chickens respond to direct progesterone treatment in a negative manner. This would agree with the possibility that progesterone is involved in termination of the preovulatory surge of LH discussed in SECTION 3.3.4.

In common with mammals (Lloyd & Karavolas, 1975; Lieberburg *et al.*, 1977; Dubois *et al.*, 1978; Sar & Stumpf, 1979; Thieulant & Duval, 1985; Krey & McGinnis, 1990), the anterior pituitary gland of birds contain binding sites for androgens (Stern, 1972; Gasc *et al.*, 1979, 1980; Kawashima *et al.*, 1989). The pituitary gland of avian species also contain progesterone binding sites (Kawashima *et al.*, 1980; Stumpf *et al.*, 1983; Sterling *et al.*, 1984a, 1987), as does the pituitary gland of mammals (Lloyd & Karavolas, 1975; Fox *et al.*, 1990). The gonadotroph cells of the rat pituitary gland show the greatest affinity for androgens (Lloyd & Karavolas, 1975; Dubois *et al.*, 1978; Sar & Stumpf, 1979; Thieulant & Duval, 1985; Fox *et al.*, 1990) and progesterone (Lloyd & Karavolas, 1975; Fox *et al.*, 1990). Specific binding of these steroids to the gonadotroph cells of birds has not been established. However, in view of the suppressive effects of testosterone and progesterone on pituitary LH, it is likely that receptors for these steroids also exist in the gonadotroph cells of the chicken.

Testosterone, progesterone and 17ß-oestradiol reduced the GnRH-I and K^+-releaseable LH and the total cellular LH with different potencies, which could suggest that these steroids exert their actions through different mechanisms. The pituitary gland is reported to contain aromatase, and 5α and 5ß-reductase activity to convert testosterone into respectively 17ß-oestradiol, and 5α-dihydrotestosterone (5α-DHT) and 5ß-DHT (see SECTION 1.3.2). Also, testosterone can be metabolised by the avian pituitary gland to androstenedione and 5ß-androstane-3α,17ß-diol (Davies *et al.*, 1980; Massa & Sharp, 1981). Of these metabolites of testosterone, only injections of 17ß-oestradiol, 5α-DHT or androstenedione reduce the concentration of plasma LH in castrated male quail (Davies *et al.*, 1980), and only 17ß-oestradiol and 5α-DHT in castrated cockerels (Massa & Sharp, 1985); androstenedione (juvenile cockerels - Wilson *et al.*, 1983), 5ß-DHT (juvenile cockerels - Massa & Sharp, 1985) and 5ß-androstane-3α,17ß-diol are without effect on plasma LH in castrated male quail (Davies *et al.*, 1980). It is not certain whether there are two distinct androgen receptors for testosterone and 5α-DHT (reviewed by Sheridan, 1991). Consequently, there are several steroids including testosterone itself, which could mediate the different inhibitory actions of testosterone on the function of gonadotroph cells from juvenile chickens.

Compared with testosterone, the metabolism of progesterone by the pituitary gland of the chicken is poorly understood. In the rat, the actions of progesterone are thought to be mediated by the formation of the more potent steroid, 5α-DHP from progesterone in pituitary and brain tissues (Batra & Miller, 1985; Mahesh & Brann, 1992). The pituitary gland of the laying hen can convert progesterone to 5α-pregnane-3,20-dione (5α-DHP) and 5ß-DHP, of which only 5α-DHP increases plasma LH in the laying hen, though less effectively than progesterone itself (Sharp & Massa, 1980). It is probable therefore that progesterone itself is the active hormone and acts to inhibit gonadotroph function.

The effects of 17ß-oestradiol are unlikely to be mediated through its metabolites. Together with the stereospecific nature of 17ß-oestradiol action, this means that its effects on the gonadotroph cell are mediated directly through 17ß-oestradiol acting on oestrogen-receptors.

The present results suggest that steroids probably do not act directly on the GnRH-receptors of gonadotroph cells *in vitro*. King *et al.* (1989) found that although 17ß-oestradiol, testosterone and progesterone reduce the maximum GnRH-I-stimulated release of LH from cultures of chicken gonadotroph cells, the steroids do not affect the ED_{50} of GnRH-I on LH release. This was confirmed in this chapter. Thus the IC_{50} values for the inhibitory action of progesterone, testosterone and 17ß-oestradiol on the GnRH-I-stimulated release of LH from pituitary cells from juvenile chickens were 3.8, 1.4 and 0.08 nM respectively, with a reduction in the maximum release to 50% of the control response in each case. These IC_{50} values are similar to the respective values of 1, 2 and 0.07 nM for progesterone, testosterone and 17ß-oestradiol reported by King *et al.* (1989). This means that treatment with steroids does not change the GnRH-receptor number or the affinity of GnRH-I in cultures of chicken pituitary cells (King *et al.*, 1989). Consequently in adult cockerels treated with oestradiol benzoate *in vivo*, the reduction in the LH peak and the amount of LH released by GnRH-I (AUC; FIGURES 5.2 and 5.3) was probably not due to an effect of the steroid on pituitary *sensitivity* to GnRH-I, but rather to a suppressive effect of the steroid on the *responsiveness* of the pituitary gland to GnRH-I. Recently however, Kawashima *et al.* (1992a) found steroid-dependent and physiological changes in GnRH-receptors in pituitary glands of adult hens, but these observations cannot be discussed with confidence for the reasons detailed in CHAPTER 4. Thus although a sensitive method for measuring GnRH-receptors in the chicken pituitary gland remains to be established, based on the evidence presented here it is unlikely that steroids directly change the characteristics of GnRH-receptors.

Studies using cultures of mammalian pituitary cells show that long-term treatment with 17ß-oestradiol (4-days) reduces the ED_{50} of GnRH on LH release (Drouin *et al.*, 1976; Tang *et al.*, 1982a) and this is associated with an increase in the number of GnRH binding sites (Tang *et al.*, 1982a) and GnRH receptor-mRNA (Sealfon *et al.*, 1990). However, short-term (<4-hours) treatment with 17ß-oestradiol increases the ED_{50} of GnRH and reduces the number of available GnRH receptors (Emons *et al.*, 1988). Furthermore, 5α-DHT reduces both the release of LH and the number of pituitary binding sites for GnRH (Giguere *et al.*, 1981). The physiological importance of this steroid-GnRH receptor relationship is also suggested by the changes in GnRH receptor number during the oestrous cycle (Clayton *et al.*, 1985). However this may be a consequence of the changes in GnRH secretion during the rat oestrous cycle, because GnRH can also up and down-regulate its own receptors both *in vivo* and *in vitro* (reviewed by Catt *et al.*, 1985; Clayton *et al.*, 1985; Clayton, 1989). This indirect action of gonadal steroids on GnRH-receptors through a change in GnRH secretion from the hypothalamus may explain the sex difference in sensitivity to GnRH-I of pituitary glands from adult chickens. In this respect, gonadectomy increases the sensitivity to GnRH of pituitary glands from juvenile hens (Wilson, 1975).

6.4 SUMMARY

A culture procedure was established which yielded functional gonadotroph cells from juvenile but not adult chickens. The gonadotroph cells from sexually immature chickens were responsive to GnRH-I and their function was reduced by the presence of phenol red in the culture medium. This effect is probably due to the oestrogenic property of phenol red.

Testosterone, progesterone and 17ß-oestradiol showed different potencies with respect to their relative abilities to suppress the GnRH-I and K^+-releaseable LH, and the total cellular content of LH. These effects of testosterone could be mediated through the various metabolites of the steroids produced by the avian pituitary gland. In contrast, progesterone is most likely the active hormone, and the actions of 17ß-oestradiol have a stereospecific requirement and appear to be mediated directly through oestrogen-receptors. This action of 17ß-oestradiol probably simulates the suppressive effect of the increasing concentrations of plasma 17ß-oestradiol on the gonadotroph cell during sexual maturation of the hen. Consequently after sexual maturation, the pituitary gland contains less LH available for release and therefore is less LH responsive to GnRH-I. Gonadal steroids did not however directly affect the sensitivity of gonadotroph cells to GnRH-I, which could suggest either an insufficient period of treatment with steroid or that an extrapituitary mechanism is involved.

7 Sexual Differences in the Intracellular Signalling Pathways for GnRH-I-Stimulated LH Release

7.1 Introduction

In CHAPTERS 3 and 4 it was observed both *in vivo* and *in vitro* that GnRH-I stimulates LH release from pituitary glands from adult cockerels in a spike-plateau pattern, whereas pituitary tissue from laying hens secrete LH in a plateau without a spike phase. Since the mechanism for GnRH-stimulated LH release involves a signal transduction pathway, a sex difference in this pathway could account for the different adult patterns of LH secretion seen *in vitro*. A spike-plateau pattern of LH release occurs after GnRH-I stimulation of pituitary cells *in vitro* from juvenile chickens (Smith *et al.*, 1987; Davidson *et al.*, 1988; SECTION 6.2.1) or adult rats (Bourne & Baldwin, 1980; Badger *et al.*, 1983; Tasaka *et al.*, 1988; Stojilkovic *et al.*, 1989a). The initial component of this response depends on mobilisation of intracellular stores of Ca^{2+} and the influx of extracellular Ca^{2+} through non-voltage-sensitive Ca^{2+} channels (non-VSCCs), whereas the plateau phase of secretion depends on Ca^{2+} entry through VSCCs and non-VSCCs (see SECTION 1.6.4.1). It therefore seems possible in laying hens that the absence of an initial spike

of LH release after GnRH-I stimulation is due to sexual differentiation of the intracellular or extracellular Ca^{2+} requirements for LH release from gonadotroph cells.

Proteins in the cell membrane of gonadotroph cells also form a part of the stimulus-secretion coupling pathway through which GnRH stimulates gonadotrophin secretion (SECTION 1.6). Alterations in the fluidity of the cell membrane may therefore influence gonadotroph function, as demonstrated by the effect of fluidity of rat pituitary cell membranes on GnRH-receptors and gonadotroph function (Gorospe & Conn, 1987a, 1987b, 1988). Membrane fluidity describes the physical state of the cell membrane, and is determined by the distribution, orientation and proportions of lipids within the membrane layer (Shinitzky, 1984; Donner *et al.*, 1990). Alteration in the fluidity of the cell membrane could therefore change the function of these proteins to alter the biological characteristics of the cell; for example it may affect the membrane potential, ion channels, transport processes, enzyme activities and receptor-binding characteristics (reviewed by Shinitzky, 1984).

This chapter describes studies on the extracellular Ca^{2+} dependency of GnRH-I-stimulated LH secretion, and the entry of Ca^{2+} through voltage-sensitive Ca^{2+} channels in pituitary glands of adult cockerels and laying hens. A study was also made to compare the membrane fluidity of the pituitary cells from adult chickens, and to determine whether steroids can affect membrane fluidity.

7.2 Results

7.2.1 Sex difference in the extracellular Ca^{2+}-dependency of LH secretion in response to GnRH-I

The basal release of LH from pituitary tissues of adult and juvenile chickens of both sex increased by 2 - 4-fold when perifused with Earle's Balanced Salt Solution (EBSS) containing 2% newborn calf serum (NBCS) and 0.5 mM EGTA, in the absence of Ca^{2+} (FIGURE 7.1 and 7.2). The secretion of LH returned to its original baseline when the tissues were perifused with EBSS containing Ca^{2+} (FIGURE 7.2).

GnRH-I stimulated a spike-plateau pattern of LH secretion from pituitary tissue of juvenile males and females maintained in medium containing Ca^{2+}, but induced only a small increase in LH release in the absence of extracellular Ca^{2+}. This coincided temporally with the spike phase of the response in the presence of extracellular Ca^{2+}, and was not associated with a second phase of secretion (FIGURE 7.1). This pattern of GnRH-I-induced LH release in the absence of extracellular Ca^{2+} was also seen using pituitary tissue from adult cockerels (FIGURE 7.2). However, removal of extracellular Ca^{2+} completely abolished any LH response to GnRH-I from pituitary tissue from laying hens.

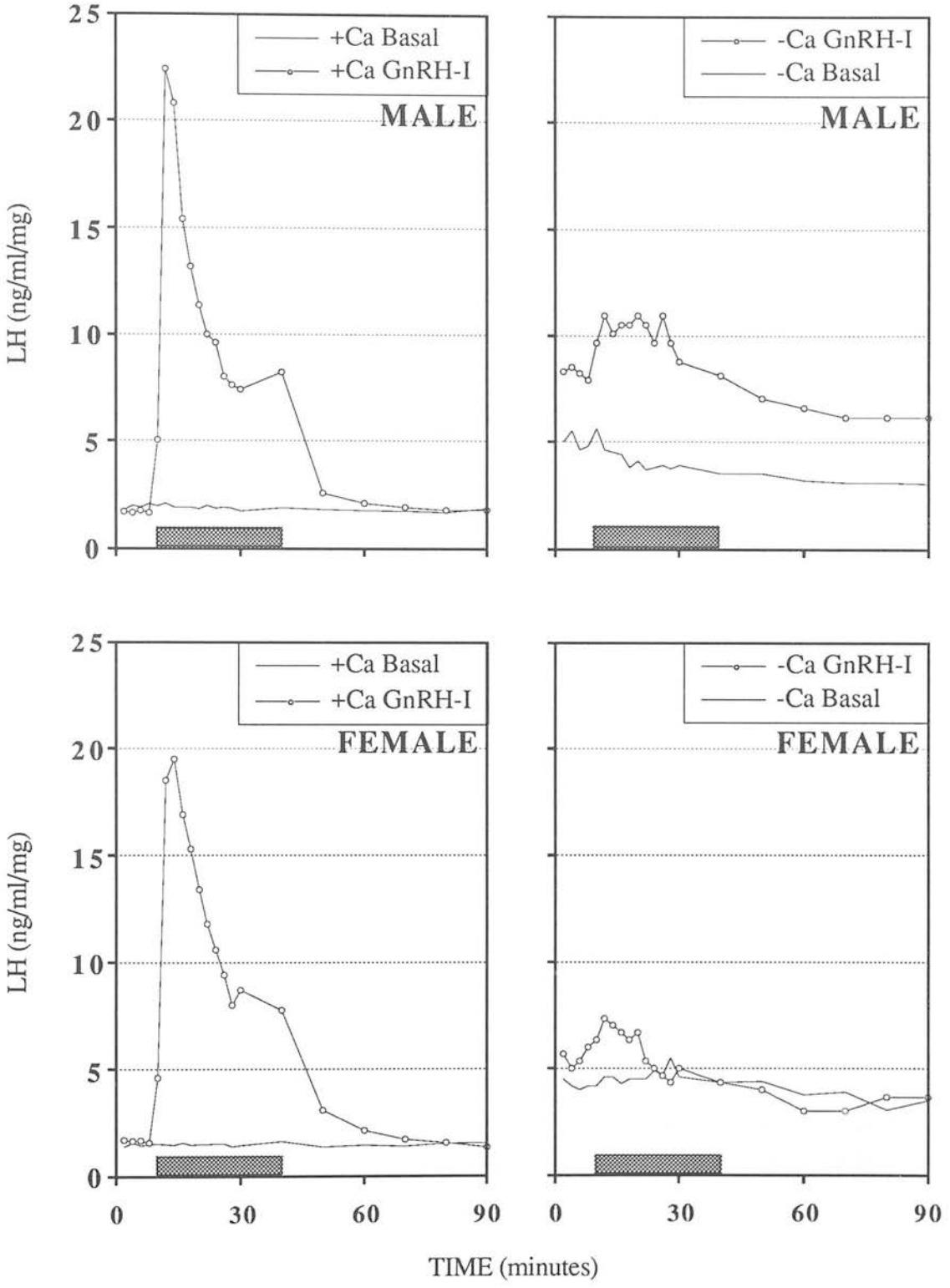


FIGURE 7.1: Effect of extracellular Ca^{2+} on GnRH-I-stimulated release of LH from pituitary tissue from juvenile males and females.
Pituitary tissues were stimulated with 100 nM GnRH-I (shaded bar) in EBSS ± Ca^{2+}. Perifusions in Ca^{2+}-free EBSS were initiated 10-min before GnRH-I stimulation. Similar results were obtained in an identical experiment.

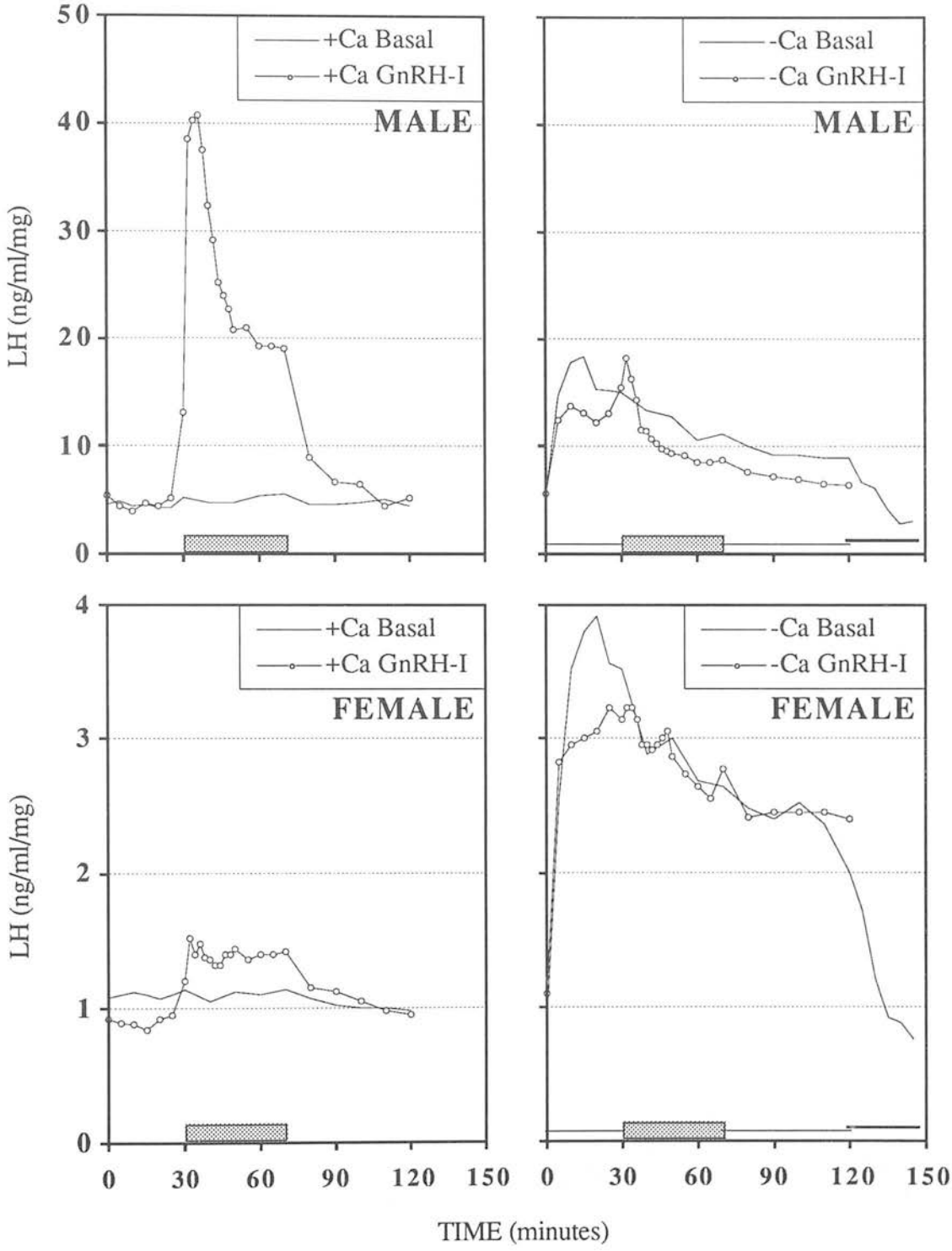


FIGURE 7.2: Effect of extracellular Ca^{2+} on GnRH-I-stimulated release of LH from pituitary tissue from adult males and females.
See FIGURE 7.1 for legend. Ca^{2+}-free medium from T = 0 - 120-min where appropriate. Grey bar = period of 100 nM GnRH-I infusion; black bar = $+Ca^{2+}$ medium. Similar results were obtained in an identical experiment. Note different scales on y-axes.

7.2.2 Role of voltage-sensitive Ca^{2+} channels in GnRH-I-stimulated LH release from pituitary glands from adult chickens

This study was repeated on 3 occasions using 2 - 3 pituitary tissues per treatment. Owing to the large variation in the basal concentrations of LH, each perifusion was expressed as a percentage of its respective basal LH concentration, calculated from the average of the first 3 samples in the absence of nifedipine. Nifedipine (NFP) alone did not affect the unstimulated release of LH from male and female pituitary tissues (FIGURE 7.3). The LH response to GnRH-I of control pituitary glands from adult cockerels and laying hens comprised an inital spike and plateau phase (FIGURE 7.3). The plateau phase of LH release from pituitary tissue of the adult cockerel and laying hen was reduced ($P<0.001$) by NFP to respectively $40 \pm 6\%$ and $42 \pm 2\%$ of the control response (not significantly different from each other). The maximum concentration of the initial LH spike was not affected by NFP for pituitary glands of both sex (TABLE 7.1). However, NFP also reduced a component of the spike phase of LH secretion from pituitary tissue of adult cockerels, but not from the spike phase of laying hens. Instead, a pronounced spike phase of LH secretion was revealed in the female response after treatment with NFP. The LH responses of both sex returned to basal levels on withdrawal of GnRH-I. The effects of NFP on the release of LH are summarised in TABLE 7.1.

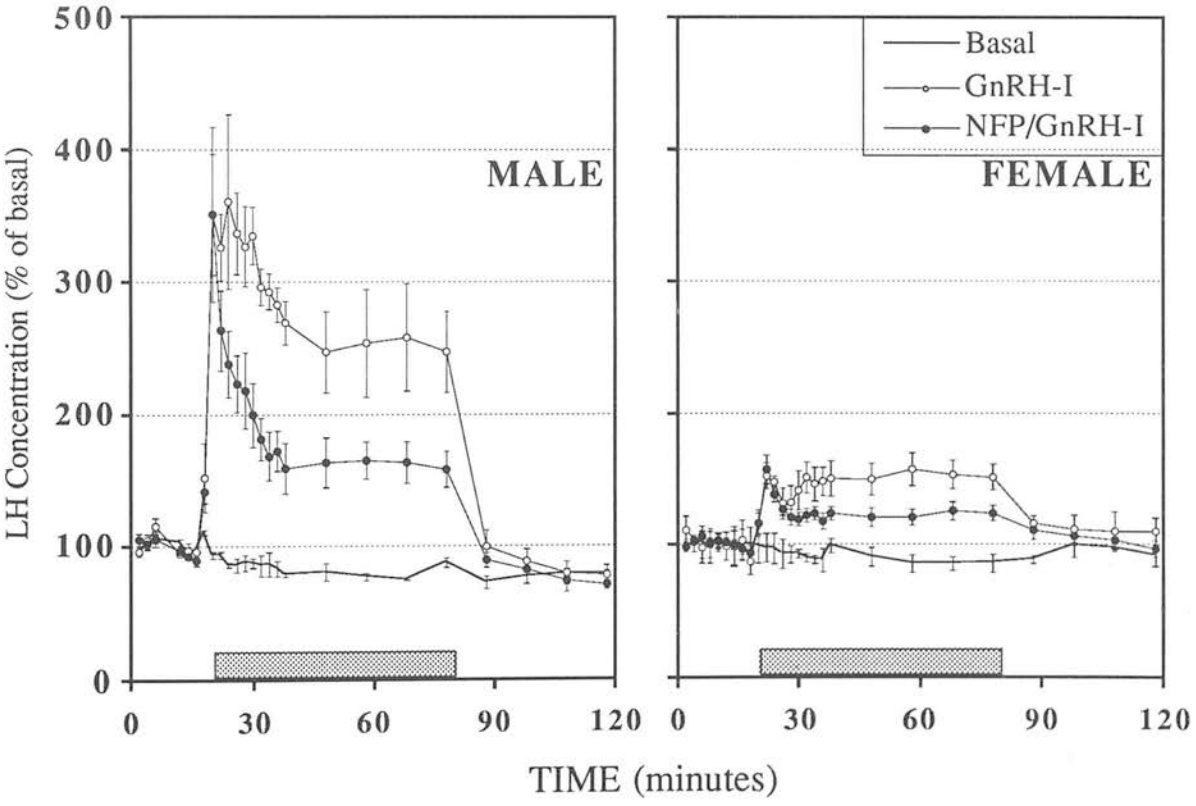


FIGURE 7.3: Effect of nifedipine on the profile of GnRH-I-stimulated LH release from adult male and female pituitary tissue.
Pituitary tissues from adult chickens (n = 6 - 8 per sex) were perifused in the presence and absence of 10 nM GnRH-I (grey bar) for 60-min with or without 20 μM nifedipine (NFP). NFP was present from Time = 10 - 120-min in the basal control and GnRH-I test perifusions. Results are expressed as a percentage of basal samples 1 - 3 (in absence of NFP).

TABLE 7.1: Effect of nifedipine on the GnRH-I-stimulated spike and plateau phase of LH secretion from adult male and female pituitary tissue.

	MALE		FEMALE	
	Spike	Plateau	Spike	Plateau
	(% of basal LH)		(% of basal LH)	
GnRH-I	351 ± 66	255 ± 17	152 ± 10	152 ± 2
NFP/GnRH-I	351 ± 30	162 ± 7***	157 ± 15	122 ± 2***

See FIGURE 7.3 for legend. Spike: peak concentration of LH at Time = 22-min. Plateau: LH concentration between Time = 38 - 80-min. ***P<0.001 compared with respective GnRH-I control.

7.2.3 Studies on membrane fluidity of pituitary cells

The differential LH responses of the adult cockerel and laying hen to GnRH-I could be due to a difference in the fluidity characteristics of the pituitary cell membranes. This hypothesis was tested by comparing the membrane fluidity of pituitary cells from laying hens and adult cockerels by fluorescence polarimetry using 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Kuhry *et al.*, 1983), and by comparing the effects of steroids on membrane fluidity.

7.2.3.1 Membrane fluidity of pituitary cells adult pituitary glands

Anisotropy is inversely related to membrane fluidity (Shinitsky, 1984). The anisotropy of pituitary cell membranes from laying hens was higher (P<0.05) than those from adult cockerels. This trend was seen in measurements at 37 and 41°C (FIGURE 7.4).

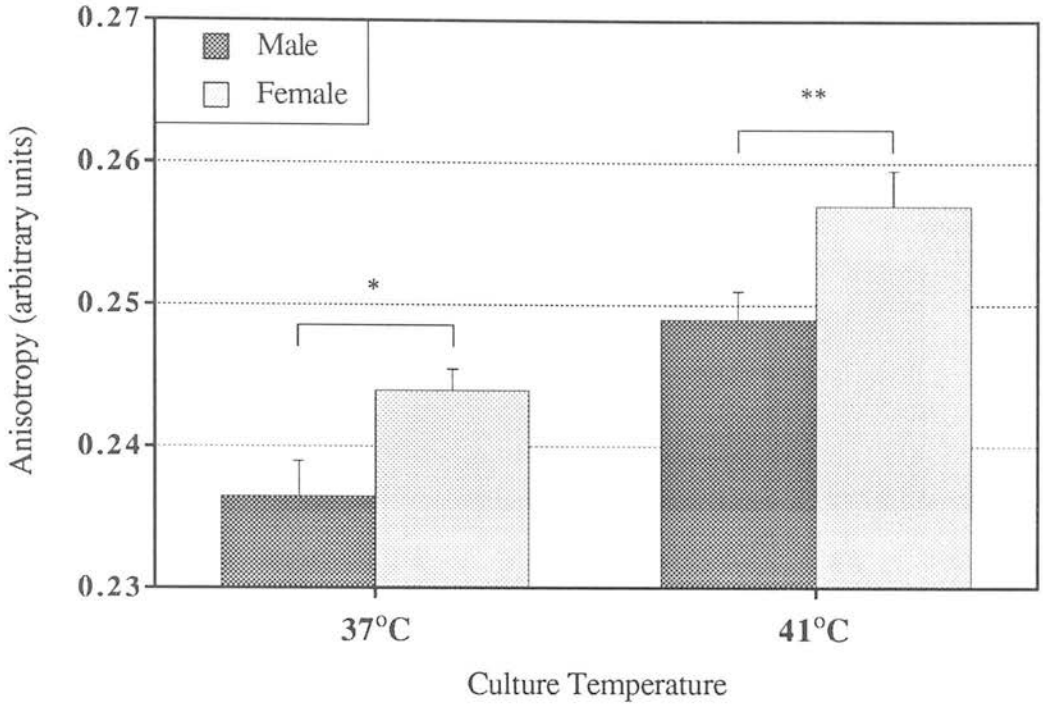


FIGURE 7.4: Anisotropy values of pituitary cells from adult cockerels and hens
Pituitary glands from age-matched adult cockerels and laying hens (n = 8 per sex) were dispersed (250,000 cells/ml) and immediately loaded with TMA-DPH for anisotropy measurements (arbitrary units) at 37 and 41°C. *P<0.05 and **P<0.01.

7.2.3.2 Effect of steroids on membrane fluidity

The high concentration of circulating 17ß-oestradiol in the laying hen may act on the pituitary gland to increase the anisotropy (and therefore reduce the fluidity) of the cell membranes. Pituitary glands from the juveniles are exposed to an environment of lower concentrations of plasma steroids than those of sexually mature chickens (Tanabe *et al.*, 1979, 1981; CHAPTER 1). The effect of gonadal steroids on the anisotropy of pituitary cells from juvenile chickens was therefore investigated.

Generally, all steroids (10 μM) increased the anisotropy of 24 and 48-hour cultures of pituitary cells from juvenile chickens (P<0.05 compared with the controls; FIGURE 7.5); the one exception was for the cells incubated with progesterone for 24-hours. Concentrations of 17ß-oestradiol below 10 μM did not significantly affect anisotropy, however the anisotropy was not altered by its stereoisomer 17α-oestradiol at any of the concentrations tested (FIGURE 7.6).

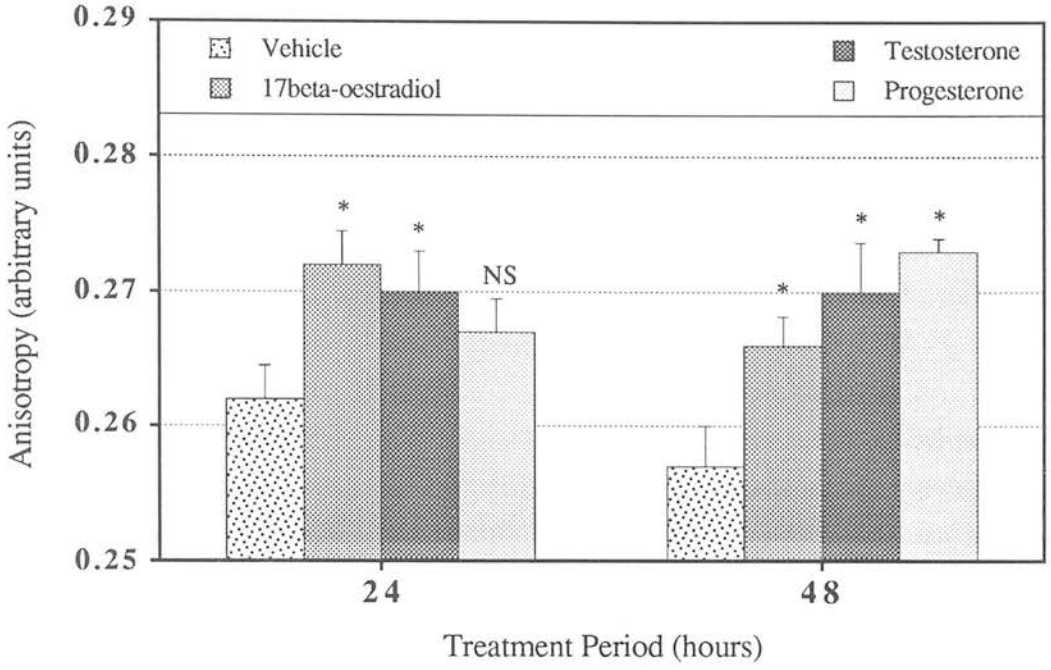


FIGURE 7.5: Effect of steroids on the anisotropy of pituitary cell membranes.
Pituitary cells from juvenile chickens were cultured for 24 or 48-h with vehicle (0.1% v/v ethanol), or 10 μM 17ß-oestradiol, testosterone or progesterone before measuring anisotropy (arbitrary units) at 37°C (500,000 cells/ml). NS = not significantly different, *P<0.05 compared with the time-matched control, n = 6.

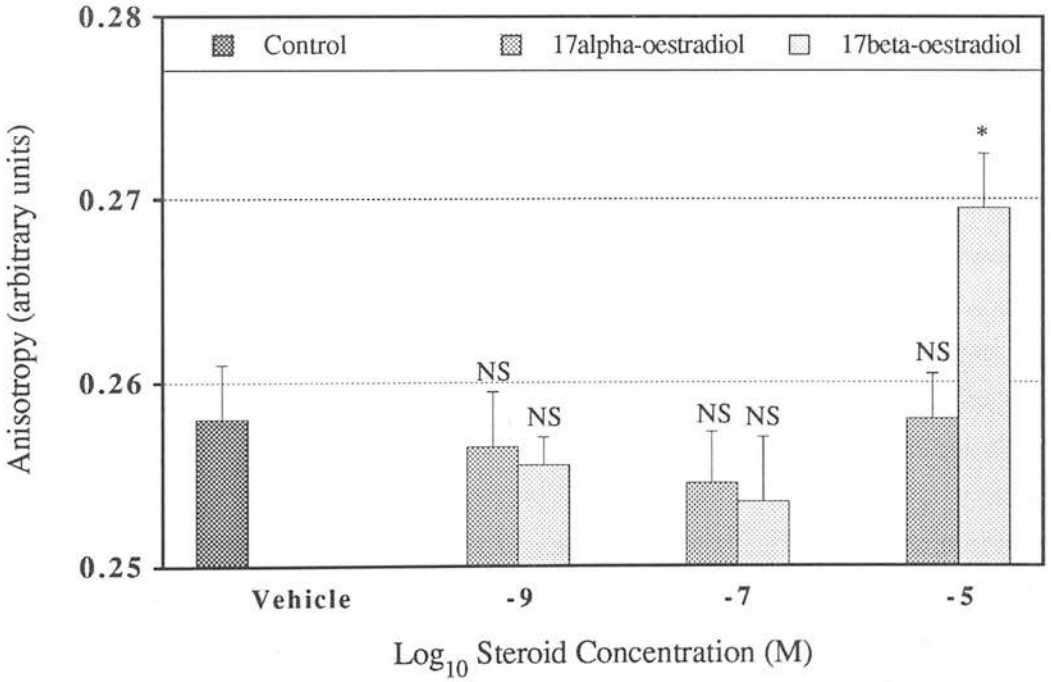


FIGURE 7.6: Comparison of the concentrations of 17α-oestradiol and 17ß-oestradiol on the anisotropy of pituitary cells.
Pituitary cells from juvenile chickens were cultured for 48-h in the presence and absence of 17α-oestradiol or 17ß-oestradiol before measuring the anisotropy (arbitrary units) at 37°C (500,000 cells/ml). NS = not significantly different, *P<0.05 compared with vehicle control, n = 9 - 14.

7.3 DISCUSSION

The results show that, as in juvenile males and females, GnRH-I stimulates a spike and a plateau phase of LH secretion from pituitary glands from adult chickens of both sex. The pituitary response of the adult female to GnRH-I was described previously as a single phase of LH secretion (see SECTION 4.2.5.2) however the demonstration of a distinct peak of LH release in the presence of nifedipine shows that the response of the adult female is in fact biphasic. Compared with the LH response of the adult cockerel, the magnitude of the spike of secretion from the laying hen is much lower, relative to the plateau phase of release.

A smaller spike of LH release was induced by GnRH-I from pituitary tissues from adult cockerels and juvenile males and females maintained in Ca^{2+}-free medium, compared with the spike phase of LH release in medium containing Ca^{2+}. In the absence of extracellular Ca^{2+}, GnRH-I failed to release LH from pituitary tissue from laying hens. Thus in terms of the requirements for extracellular Ca^{2+}, the first component of GnRH-I-induced LH release is sexually differentiated in adult but not juvenile chickens. LH secretion in response to GnRH-I therefore depends largely on extracellular Ca^{2+} but an extracellular Ca^{2+}-independent component also operates during the spike of LH release from pituitary tissues of adult cockerels and juveniles of both sex. Similar deductions, with respect to the Ca^{2+} requirements for LH secretion, have been made from studies using pituitary tissue from juvenile chickens (Smith *et al.*, 1987; Davidson *et al.*, 1988), rats (Smith *et al.*, 1987; Bourne *et al.*, 1988; Chang *et al.*, 1988c; Hansen *et al.*, 1988; Tasaka *et al.*, 1988; Stojilkovic *et al.*, 1992b), and fish (Levavi-Silvan & Yaron, 1989; van Asselt *et al.*, 1989; Chang *et al.*, 1990; Jobin & Chang, 1992).

There is a precedent for sexual differentiation of dependency on extracellular Ca^{2+} for LH release in the rat. The pituitary gland of the adult *male* is reported to depend wholly on extracellular Ca^{2+} in order to release LH in response to GnRH (Bourne, 1988; Bourne *et al.*, 1988). The oestrogen-dependency of this difference is demonstrated by the ability of 17ß-oestradiol to reproduce a female-type LH response in male rats, and the fact that the loss of the extracellular Ca^{2+}-independent component of LH secretion from pituitary tissue from ovariectomised rats can be reinstated by oestrogen-replacement (Baldwin *et al.*, 1983; Evans *et al.*, 1983; Bourne *et al.*, 1988). The sex difference in the extracellular Ca^{2+}-dependent component of LH release from pituitary glands of adult chickens may also be related to the sexually differentiated concentrations of circulating 17ß-oestradiol. The oestrogen-dependent responses of the rat are a component of the direct positive feedback effects of 17ß-oestradiol on LH release. In contrast, 17ß-oestradiol only appears to exert an inhibitory action on LH release from the pituitary gland of the chicken (see SECTION 1.5.2). Thus, 17ß-oestradiol may abolish the extracellular Ca^{2+}-independent component of GnRH-I-induced LH release from pituitary tissue from laying hens. No experiments were carried out to examine whether the extracellular Ca^{2+}-dependent release of LH is affected by treatment with 17ß-oestradiol. However when pituitary tissue from adult cockerels was incubated with 17ß-oestradiol *in vitro*, the GnRH-I-stimulated plateau phase of LH was significantly

increased without affecting the magnitude of the spike phase of release (FIGURE 5.7, TABLE 5.2). Consequently, the effect of 17ß-oestradiol on the plateau phase of LH secretion indicates a specific action on a portion of the stimulus-secretion coupling mechanism involved in this phase of GnRH-I-stimulated LH release. The absence of an effect of 17ß-oestradiol on the magnitude of the LH spike *in vitro* could mean that the loss of the extracellular Ca^{2+}-dependent phase of LH secretion is mediated through an influence outside the pituitary gland, by perhaps the hypothalamus, or more likely that a longer period of oestrogen-treatment is required to suppress this phase of secretion (see SECTION 5.3).

The mechanisms involved in intracellular signalling for GnRH-stimulated release of LH and the ways in which 17ß-oestradiol modify these, are reviewed in SECTION 1.6. Different intracellular signalling pathways are involved during the spike and plateau phases of LH secretion from the pituitary gland in response to GnRH. These include phase-dependent differences in the relative requirements for extracellular and intracellular Ca^{2+} (Limor *et al.*, 1987; Chang *et al.*, 1988c; Davidson *et al.*, 1988; Stojilkovic *et al.*, 1992b), arachidonic acid (Chang *et al.*, 1987) and protein kinase C (Chang *et al.*, 1987; Fahmy *et al.*, 1989; Waters & Conn, 1992).

The role of extracellular Ca^{2+} in the LH response to GnRH-I was investigated in pituitary tissue from adult chickens using the L-type Ca^{2+} channel blocker nifedipine (NFP). NFP markedly reduced the release of LH from pituitary tissue from both sex during the plateau phase, without affecting the amplitude of the GnRH-I-induced spike of secretion, and the later part of the spike phase from pituitary tissue from adult cockerels was also absent during treatment with NFP. These observations indicate the importance of Ca^{2+} entry through L-type Ca^{2+} channels during the second phase of LH secretion from pituitary tissue of both sex, and during the spike of LH secretion from pituitary tissue from adult cockerels. The spike phase is therefore sexually differentiated with respect to the requirement for Ca^{2+} entry through L-type Ca^{2+} channels.

The depolarisation-induced release of LH and entry of Ca^{2+} into pituitary cells, and their blockade by Ca^{2+} channel blockers, identifies the presence of depolarisation-gated (or voltage-sensitive) Ca^{2+} channels in gonadotroph cells of the chicken. There are three classes of voltage-sensitive Ca^{2+} channels (VSCC), assigned T, L and N, of which only the L-type is sensitive to blockade by NFP (Nowycky *et al.*, 1985; Miller, 1987). This activity of NFP on the GnRH-I-stimulated release of LH demonstrated here therefore indicates the functional importance of these channels in gonadotroph cells from adults of both sex, and confirms the observations in pituitary cells from juvenile chickens (Smith *et al.*, 1987; Davidson *et al.*, 1988). However NFP did not completely suppress the GnRH-I-stimulated release of LH. The incomplete blockade of the LH plateau is not due to an inadequate concentration of NFP (20 µM) in the present experiments because half this concentration (10 µM) prevents the K^+-induced release of LH from pituitary cells from juvenile chicken; a stimulus which opens VSCCs (Davidson *et al.*, 1988). This observation together with the absolute requirement for extracellular Ca^{2+} during the plateau phase of secretion suggests an influx of Ca^{2+} through NFP-*insensitive* Ca^{2+} channels (or non-L-type Ca^{2+} channel; Smith *et al.*,

1987; Davidson *et al.*, 1988; Naor *et al.*, 1988; Tasaka *et al.*, 1988; Stojilkovic *et al.*, 1992b). This mode of Ca^{2+} entry could be through a sub-class of L-type VSCC in gonadotroph cells of the rat (Blotner *et al.*, 1990) or a T-type channel (Chen *et al.*, 1989; Davidson *et al.*, 1988; Smith *et al.*, 1989), however only the latter channel has been identified in rat gonadotroph cells by electrophysiological techniques (Stutzin *et al.*, 1989; Stojilkovic *et al.*, 1992b).

Receptor-operated Ca^{2+} channels represent an alternative route for Ca^{2+} entry (Davidson *et al.*, 1988; Smith *et al.*, 1989). These Ca^{2+} channels are directly coupled to, or associated with a receptor either for the primary signal, in this case GnRH-I, or a second messenger molecule such as IP_3 (Smith *et al.*, 1989). For example, IP_3 and IP_4 co-operate to control Ca^{2+} influx into sea urchin eggs (Irvine & Moor, 1986), and IP_6 stimulates $^{45}Ca^{2+}$ entry into rat pituitary cells (Sortino *et al.*, 1990). There is however, no clear evidence for a functional role of receptor-operated Ca^{2+} channels or T-type VSCCs in the release of LH from GnRH-stimulated gonadotroph cells of the rat or chicken.

In seeking an explanation for the sex difference in the extracellular Ca^{2+}-dependency of GnRH-I-stimulated LH secretion, attention is focussed on the signalling events during the acute phase of LH secretion because it is here that a sex difference is seen both in the presence (FIGURE 4.9) or absence of extracellular Ca^{2+} (FIGURE 7.2), and in the presence of NFP (FIGURE 7.3). Since GnRH requires an increase in $[Ca^{2+}]_i$ for LH secretion to occur (Stojilkovic *et al.*, 1992b), the extracellular Ca^{2+}-independent release of LH from pituitary tissue of adult cockerels and juvenile males and females must depend upon the mobilisation of Ca^{2+} from intracellular stores. This has been shown for the rat (Limor *et al.*, 1987; Smith *et al.*, 1987; Naor *et al.*, 1988; Tasaka *et al.*, 1988; McArdle & Poch, 1992) and the chicken (Smith *et al.*, 1987; Davidson *et al.*, 1988), and is supported by the observation in rat pituitary cells that in the absence of extracellular Ca^{2+}, GnRH stimulates small increases in $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal stores, and a small release of LH (Limor *et al.*, 1987; Naor *et al.*, 1988; Tasaka *et al.*, 1988; Stojilkovic *et al.*, 1989a). The presence of a releaseable store of intracellular Ca^{2+} in pituitary cells of sexually immature chickens has been demonstrated using thapsigargin (Johnson & Tilly, 1991); a pharmacological agent which mobilises Ca^{2+} from intracellular pools (Koshiyami & Tashijian, 1991). However, IP_3 is the endogenous agent which mobilises Ca^{2+} from this labile pool (Berridge & Irvine, 1989) and GnRH stimulates the accumulation of this molecule in pituitary cells from the rat (Schrey, 1985; Andrews & Conn, 1986; Naor *et al.*, 1986; Morgan *et al.*, 1987) and the chicken (Davidson *et al.*, 1988; King *et al.*, 1989). This probably accounts for the ability of GnRH to release $^{45}Ca^{2+}$ from pituitary cells of the chicken (Davidson *et al.*, 1988; King *et al.*, 1989) and the rat (Hopkins & Walker, 1978) loaded previously with $^{45}Ca^{2+}$. This suggests that the extracellular Ca^{2+}-independent release of LH from pituitary tissue from juveniles of both sex and adult cockerels, is due to an IP_3-dependent mobilisation of Ca^{2+} from intracellular stores. However, gonadotroph cells from laying hens required extracellular Ca^{2+} in order to secrete LH, which means that GnRH-I does not stimulate, or stimulates an insufficient release of intracellular Ca^{2+}. GnRH-I-stimulated LH secretion therefore occurs by an IP_3-independent mechanism in pituitary tissue from laying hens.

The GnRH-I-stimulated spike but not plateau phase of LH secretion from pituitary glands from adult chickens is also sexually differentiated with respect to the requirement for Ca^{2+} entry through L-type voltage-sensitive Ca^{2+} channels (FIGURE 8.1). The operation of these Ca^{2+} channels is confined to the plateau phase of LH secretion from pituitary tissue from laying hens, which suggests that the activation of the channels during the spike phase is suppressed or delayed in onset. This route of Ca^{2+} influx is thought to be activated by depolarisation of the cell membrane, by activation of the receptor-G-protein-Ca^{2+} channel complex, or by an increase of intracellular Ca^{2+} concentration (reviewed by Berridge, 1993). The function of Ca^{2+} channels is regulated by phosphorylation and dephosphorylation by protein kinases (reviewed by Berridge, 1993). The function of Ca^{2+} channels are reported to be affected by 17ß-oestradiol (see SECTION 1.6.4.1), a high concentration of which may establish the sexually differentiated function of L-type Ca^{2+} channels during the spike phase of LH secretion from pituitary glands from adult chickens. This would be consistent with the observed modulatory effects of 17ß-oestradiol on gonadotroph function *in vivo* (CHAPTER 5), *in vitro* (CHAPTER 6) and on the membrane fluidity of pituitary cells (see below).

The high concentration of circulating 17ß-oestradiol in laying hens may reduce the extracellular Ca^{2+}-independent component of LH secretion (spike phase) in response to GnRH-I. Treatment with 17ß-oestradiol reduces the GnRH-I-stimulated spike and plateau phases of LH secretion from cultures of pituitary cells from juvenile chickens (King *et al.*, 1989). However this treatment does not affect the turnover of inositol polyphosphates stimulated by GnRH-I, but reduces the release of $^{45}Ca^{2+}$ from cells loaded previously with $^{45}Ca^{2+}$ (King *et al.*, 1989). This observation suggests two effects on the fluxes of Ca^{2+}. Firstly, since IP_3 releases intracellular Ca^{2+}, this indicates that 17ß-oestradiol acts at a level of signal transduction after IP_3 formation and before the mobilisation of intracellular stores of Ca^{2+}. The failure of pituitary tissue from laying hens to respond to GnRH-I in the absence of extracellular Ca^{2+} is therefore due to suppression of Ca^{2+} release from internal stores by 17ß-oestradiol. Secondly, it suggests that 17ß-oestradiol does not affect the ability of the higher inositol polyphosphates, IP_4 and IP_6, to open non-VSCCs in pituitary cells (Sortino *et al.*, 1990).

The importance of membrane fluidity in GnRH-I-stimulated LH release is suggested by the fact that the cell membrane serves many functions including structural and selective transport roles but also participates in stimulus-secretion coupling. Typically, interaction of cell membrane receptors with their ligands initiate cell activation, and this interaction is altered by changes in membrane fluidity (Heron *et al.*, 1980; Dave & Witorsch, 1984; DePietro & Byrd, 1990); for example, the rate of dimerisation and microaggregation of GnRH-receptors (Conn *et al.*, 1987) may be affected to alter the rate or efficiency of gonadotroph activation. The activation signal is transduced into the cell by G-proteins to activate enzymes which are located in or bind to the cell membrane; e.g. protein kinase C (Lester, 1990), phospholipase A_2 (Hirata *et al.*, 1980; Kuomanov *et al.*, 1990) and adenylyl cyclase (Giocondi *et al.*, 1990). Some of these enzymes convert membrane

phospholipids to second messenger molecules which then amplify the stimulatory signal in the cell. Ion fluxes through the membrane channels also contribute to cell activation (Bikle *et al.*, 1984; Gorospe & Conn, 1988). These pathways are sexually differentiated and oestrogen-dependent in the rat, with respect to GnRH-induced LH release (see SECTION 1.6.4). Finally, hormone secretion involves fusion of the membranes of the cell and secretory granule. Consequently, differences in membrane fluidity alter the final cellular response (Hirata *et al.*, 1980; Shinitsky, 1984; Kuomanov *et al.*, 1990).

The lower membrane fluidity of pituitary cells from laying hens than from adult cockerels suggests a mechanism for the sex differences in the intracellular signalling pathway for GnRH-I. Further, the reduction in the membrane fluidity of pituitary cells from juvenile chickens induced by high doses of 17ß-oestradiol suggests that the high plasma concentration of this steroid in laying hens might be responsible for the lower membrane fluidity of its pituitary cells compared with those of adult cockerels. This difference may contribute to the sexually differentiated LH responses of adult chickens to GnRH-I. However testosterone and progesterone also lowered membrane fluidity which suggests that the *concentration* of steroid could be the overriding determinant rather than the *class* of steroid itself. In this regard, doses of 17ß-oestradiol below 10 µM failed to change the membrane fluidity. A reduction of membrane fluidity by treatment with 17ß-oestradiol is also seen in human breast cancer cells (Clarke *et al.*, 1990) and suggested to be related to the partitioning of 17ß-oestradiol within the hydrophobic domains of the cell membrane (Clarke *et al.*, 1990). This mechanism of action is highly improbable in the present study because there was no change in membrane fluidity with equimolar concentrations of 17α-oestradiol. This indicates a stereospecific requirement for the oestrogen-induced reduction of membrane fluidity in pituitary cells of the juvenile chicken. A very high and therefore non-physiological concentration of 17ß-oestradiol was required to produce this effect which suggests that prolonged treatment with more moderate concentrations of 17ß-oestradiol might also produce a similar reduction in membrane fluidity.

Since LH-gonadotroph cells represent about 8% of all pituitary cell-types in the chicken (see SECTION 4.2.3) it is pertinent to ask whether or not measurements of changes in membrane fluidity of the entire population of pituitary cells induced by 17ß-oestradiol, are representative of changes in the gonadotroph population. Oestrogen-receptors are located mainly in the gonadotroph cells of the chick embryo (Gasc *et al.*, 1980) and the rat (Lloyd & Karavolas, 1975; Dubois *et al.*, 1978; Sar & Stumpf, 1979; Thieulant & Duval, 1985; Fox *et al.*, 1990), and there can be no doubt that 17ß-oestradiol interacts with the cell membrane of gonadotrophs. However, the observation that the increased release of prolactin but not of growth hormone from chicken pituitary glands incubated with 17ß-oestradiol *in vitro* (Hall *et al.*, 1984), indicates that lactotroph cells but not somatotroph cells are also oestrogen-sensitive. This is consistent with the view that the reduction in membrane fluidity of pituitary cells following treatment with 17ß-oestradiol, does not represent a decrease only in the cell membranes of gonadotrophs.

The fluidity of rat pituitary cells is increased by 17ß-oestradiol by redistributing membrane phospholipids and is suggested to participate in the oestrogen-induced potentiation of LH secretion from gonadotroph cells *in vitro* (Drouva *et al.*, 1986, 1987). This is through the 17ß-oestradiol-induced increase in methyl-transferase activity of the cells which catalyses the serial N-methylation of pituitary membrane phosphatidylethanolamine (PEA) to form phosphatidylcholine (Drouva *et al.*, 1986, 1987). These methylated products of PEA are translocated towards the outer leaf of the membrane bilayer and shift the distribution of phospholipid across the membrane to increase the fluidity characteristics of the membrane (Drouva *et al.*, 1987). Such a change in membrane fluidity can modify cell function (Hirata & Axelrod, 1978, 1980) because membrane phospholipids play key roles in stimulus-secretion coupling by providing substrates for phospholipases A_2 and C to produce arachidonic acid, IP_3 and 1,2-diacylglycerol (see SECTION 1.6). The sex difference in membrane fluidity between pituitary cells from adult chickens might produce differences in the availability of these phospholipids and the generation of second messengers, and therefore differences in the LH responses to GnRH-I.

Methyl-transferase activity is greatest in the membranes of the secretory granules and the endoplasmic reticulum of the rat pituitary gland (Drouva *et al.*, 1986). This might be important in regulating the process of secretion itself or the mobilisation of intracellular Ca^{2+} stored in an endoplasmic reticulum-like structure (see SECTION 1.6.1). In pituitary cells from juvenile chickens, the GnRH-I-induced release of LH is reduced by 17ß-oestradiol by interferring with the intracellular signalling pathway between IP_3 formation and release of Ca^{2+} from internal stores. It is possible that 17ß-oestradiol reduces the fluidity of the membranes around these stores of Ca^{2+} to prevent its mobilisation. This might be through a reduction in sensitivity of IP_3-induced Ca^{2+} release which occurs by a reduced content of Ca^{2+} in the endoplasmic reticulum-like structure, or depend on IP_3-receptor heterogeneity through post-translational modification of the receptor protein (reviewed by Berridge, 1993). The technique used in the present studies measures the membrane fluidity of *intact* cells and therefore provides no information on the fluidity of internal membranes.

Species differences between the effects of 17ß-oestradiol on gonadotroph function may explain why the fluidity of pituitary cell membranes is increased by 17ß-oestradiol in rats, but reduced in those of the chicken. In the rat, 17ß-oestradiol inhibits or enhances the LH response of gonadotroph cells to GnRH stimulation (CHAPTER 1), but only the *stimulatory* effect of 17ß-oestradiol on LH secretion has been studied (Drouva *et al.*, 1986, 1987). Only the inhibitory effect of 17ß-oestradiol is found in GnRH-I-stimulated gonadotroph cells from juvenile chickens (King *et al.*, 1989; SECTION 6.2.3), and this inhibition is correlated with a reduction in membrane fluidity. It is possible that this relationship between gonadotroph function and membrane fluidity is involved in the suppressive action of 17ß-oestradiol through the mechanisms described above.

7.4 SUMMARY

GnRH-I stimulated a spike-plateau profile of LH secretion from pituitary tissue from both sex of juvenile and adult chickens. However when maintained in a Ca^{2+}-free medium, pituitary tissues from juveniles of both sex and from adult cockerels but not from laying hens, released LH in response to GnRH-I. This sexually differentiated extracellular Ca^{2+}-independent component of LH secretion corresponded temporally with the spike phase of release. It is concluded that the GnRH-I-induced mechanism of LH release from pituitary tissue from laying hens is independent of the mobilisation of intracellular Ca^{2+}, and that entry of Ca^{2+} during the spike phase of LH secretion occurs through non-L-type Ca^{2+} channels. Both non-L-type and L-type Ca^{2+} channels operate during the plateau phase of secretion. In contrast, the spike phase of LH release from pituitary tissue from adult cockerels involves three modes of Ca^{2+} flux, comprising an intracellular Ca^{2+}-dependent component and Ca^{2+} entry through L-type and non-L-type Ca^{2+} channels. The plateau phase of LH secretion only requires the two latter routes of Ca^{2+} flux.

The lower membrane fluidity of pituitary cells from laying hens than those from adult cockerels could depend on a stereospecific effect of 17ß-oestradiol. The apparent maturational loss of the IP_3-dependent mechanism of LH secretion from pituitary glands of hens may be related to the high concentration of plasma 17ß-oestradiol in laying hens. Since 17ß-oestradiol reduces the mobilisation of intracellular Ca^{2+} without affecting the turnover of inositol polyphosphates in pituitary cells from juvenile chickens, a reduction in membrane fluidity of the endoplasmic reticulum-like structures which store Ca^{2+} by 17ß-oestradiol may suppress the IP_3-induced release of intracellular Ca^{2+}.

It is concluded that the sex difference in profile of GnRH-I-stimulated LH release from pituitary glands from adult chickens, is due to differences in the intracellular signalling mechanism by which LH is released during the spike and plateau phases. These differences could be mediated through the higher concentrations of plasma 17ß-oestradiol in laying hens than in adult cockerels. Specifically, 17ß-oestradiol may suppress the IP_3-dependent component of the intracellular signalling pathways involved in LH secretion through its effect to reduce the membrane fluidity of gonadotroph cells.

8 Summary and General Discussion

A comparison of the sex differences in the sensitivity and responsiveness of adult chickens to GnRH-I *in vivo* and *in vitro* (CHAPTERS 3 and 4) showed that they are functions of the anterior pituitary gland. The increased duration of the LH response to GnRH-I observed after the onset of sexual maturation in hens *in vivo* (CHAPTER 3) was not observed *in vitro* (CHAPTER 4) and therefore depends on an extrapituitary mechanism. These sex differences only became fully established after the onset of sexual maturation but the LH responses to GnRH-I were similar in adult cockerels and juveniles of both sexes (CHAPTER 3). It is therefore concluded that the major factor most likely to account for the sexually differentiated LH responses to GnRH-I in adults is the increase in plasma 17ß-oestradiol at the onset of sexual maturation in hens.

Unlike mammals, in which 17ß-oestradiol exerts both stimulatory and inhibitory effects on the hypothalamic-gonadotroph axis, in the chicken, 17ß-oestradiol exerts principally an inhibitory action. The sexually differentiated LH response of adult chickens to GnRH-I is therefore considered to be an aspect of the mechanism of the inhibitory action of 17ß-oestradiol. This might be exerted at the level of the hypothalamus on GnRH-I synthesis and/or release, or at the level of the anterior pituitary gland on the synthesis and/or release of LH. The higher concentration of hypothalamic GnRH-I in adult than juvenile chickens (CHAPTER 3) supports the view that the onset of sexual maturation is induced by an increased 'hypothalamic drive' to stimulate gonadal growth and secretion of steroids. It follows that sexual differentiation of the concentrations of hypothalamic GnRH-I in adult but not juvenile chickens (CHAPTER 3), is consistent with an inhibitory action of 17ß-oestradiol in the hen at the level of the hypothalamus superimposed upon this increased 'hypothalamic drive'. It is difficult to accept that this inhibitory action reduces the concentration of plasma LH in laying hens to below those in juvenile hens by reducing the release of GnRH-I from the hypothalamus of laying hens to less than that from the hypothalamus of juvenile hens. This is because the increase in content of hypothalamic GnRH at the onset of sexual maturation in the hen is correlated temporally with a decrease in pituitary responsiveness or sensitivity to administered GnRH (Knight *et al.*, 1985). If it is argued that 17ß-oestradiol suppresses GnRH-I release in the laying hen to below that in juvenile hens, it must be concluded that the increase in FSH secretion required to sustain ovarian follicular growth is controlled by another mechanism, possibly another gonadotrophin-releasing hormone. Although GnRH-II is an obvious candidate as an FSH-releasing hormone and the concentration of hypothalamic GnRH-II was greater in hens than in cockerels after, but not before, the onset of sexual maturation (CHAPTER 3), the balance of evidence suggests that, in physiological circumstances, GnRH-II does not act directly on the anterior pituitary gland (Sharp *et al.*, 1990).

It is therefore concluded that the major focus for the sexually differentiated LH response to GnRH-I is the LH-gonadotroph cell, and is mediated by 17ß-oestradiol. A model, based upon observations reported in CHAPTERS 3 - 7, is proposed to account for some of the sex differences in the mechanism of GnRH-I-induced LH release from the LH-gonadotrophs of adult chickens (FIGURE 8.1). This model is discussed in relation to two questions. Firstly, are the present

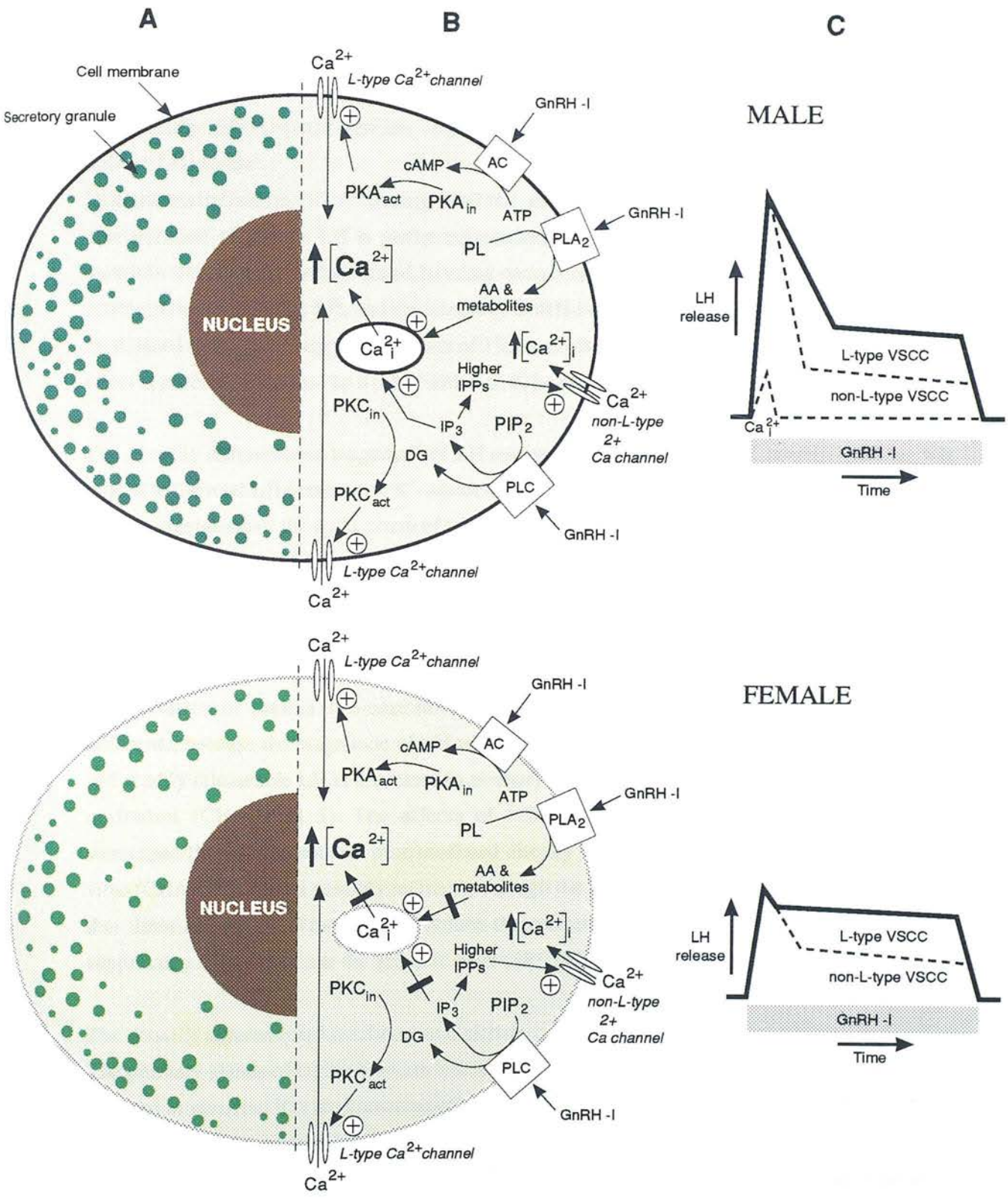


Figure 8.1: Model to explain the sex difference in the mechanism of GnRH-I-induced release of LH from gonadotrophs of adult chickens. Diagramatic representation of (A) the distribution and quantity of secretory granules in LH-gonadodotroph cells. (B) the intracellular signalling mechanisms, and (C) the calcium-dependency of LH secretion during the initial and sustained phases of LH secretion. AA=arachidonic acid, AC=adenlyl cyclase, ATP=adenosine triphosphate, cAMP=cyclic 3'5'-adenosine monophosphate, DG=1,2-diacylglycerol, IP3=inositol 1,4,5-trisphosphate, IPPs=inositol polyphosphates, PIP2=phosphatidylinositol 4,5-bisphosphate, PKA and PKC=protein kinases A and C (active or inactive, PL=phospholipid, PLA2 and PLC=phospholipases A2 and C, VSCC= voltage-sensitive calcium channel. ⊁ blocked by 17β-oestradiol action. ····· low membrane fluidity, —— high membrane fluidity. ⊕ Stimulation. GnRH-receptors and G-proteins are not shown for the sake of clarity.

observations consistent with a direct effect of 17ß-oestradiol on the pituitary gland, and secondly, are the effects of 17ß-oestradiol on the LH response and sensitivity of the pituitary gland to GnRH-I attributable to mechanisms which are independent of a depressed concentration of pituitary LH?

The sexually differentiated baseline concentrations of plasma LH and magnitude of GnRH-I-induced LH release

Immunoneutralisation of circulating GnRH-I in adult chickens suggested that the resting concentration of plasma LH is partly independent of GnRH-I, especially in laying hens, and therefore the sexually differentiated baseline concentrations of plasma LH are functions of the concentration of pituitary LH, and the release of GnRH-I (CHAPTER 3). These sex differences are established through the suppressive effect of 17ß-oestradiol in laying on pituitary LH content. This effect is probably mediated by a direct action of 17ß-oestradiol on LH synthesis.

The sexually differentiated magnitude of LH release in response to GnRH-I in adult chickens was related to the total LH content and K^+-releaseable store of LH in the pituitary gland (CHAPTER 4) and the magnitude of the spike phase of LH secretion (see below). There was no sex difference in the proportion of LH-gonadotroph cells (SECTION 4.2.3). Measurements of the number of secretory granules in gonadotroph cells from adult chickens showed that there were fewer in cells from laying hens than from adult cockerels (CHAPTER 4; FIGURE 8.1). It is therefore concluded that gonadotroph cells from laying hens contain less LH than those from adult cockerels. The high concentration of plasma 17ß-oestradiol in laying hens is probably responsible for this sex difference because the magnitude of LH release in response to GnRH-I is reduced, and the total and readily releaseable LH is depressed in pituitary glands from adult cockerels treated with 17ß-oestradiol (CHAPTER 5). The effects of 17ß-oestradiol on pituitary LH content had a stereospecific requirement and were mediated directly on the pituitary gland, as demonstrated *in vitro* (CHAPTER 6) and possibly indirectly through the hypothalamus (CHAPTER 5). It is likely that these actions of 17ß-oestradiol reduce the concentration of LH in the pituitary gland by suppressing LH synthesis at the genomic level (SECTION 5.3).

The sexually differentiated sensitivity to GnRH-I

The pituitary glands of laying hens are less sensitive to GnRH-I than those of the adult cockerel *in vivo* and *in vitro*, but it is not known whether these differences are due to differences in the number or affinity of the receptors for GnRH-I because the attempts to characterise the pituitary GnRH-receptors of the chicken were unsuccessful (SECTION 4.2.5). However, evidence was obtained to suggest that 17ß-oestradiol directly reduces the responsiveness but not the sensitivity (ED_{50}) of pituitary cells from juvenile chickens to GnRH-I *in vitro* (SECTION 6.2.3.3). This contrasts with reports in mammals that 17ß-oestradiol affects the number of GnRH-receptors in pituitary cell cultures (SECTION 6.3.2). A more prolonged treatment of pituitary cells from chickens with 17ß-oestradiol *in vitro* may be required to change the sensitivity to GnRH-I. Alternatively, the lower sensitivity to GnRH-I of pituitary tissue from laying hens than adult cockerels may develop indirectly through an extrapituitary mechanism of 17ß-oestradiol action. The well-established effect

of 17ß-oestradiol in mammals on GnRH secretion, the autoregulatory effect of GnRH on its receptors in the pituitary gland, and the corresponding changes in sensitivity (SECTION 6.3.2) remains to be investigated in the chicken.

The sexually differentiated duration of LH secretion

Sexual differentiation of the duration of increased plasma LH in response to GnRH-I in adult chickens *in vivo* was not due to a sex difference in the plasma half-life of GnRH-I (Sharp *et al.*, 1987) or in the mechanisms for removing LH from plasma (SECTION 3.2.3). It is possible that gonadal steroids regulate the circulatory half-life of endogenous LH through their effects on post-translational processing of LH in the gonadotroph cells of the chicken (FIGURE 8.1), as they do in mammals (SECTION 3.3.4). The high circulating concentration of 17ß-oestradiol in laying hens may therefore increase the plasma half-life of LH by post-translational modification. Consequently the LH released by the pituitary gland of laying hens in response to GnRH-I, circulates in the plasma for a longer period than the LH secreted by the pituitary gland of adult cockerels. This would explain why the duration of the LH response to GnRH-I is sexually differentiated *in vivo* but not *in vitro*.

Another explanation is based on the stimulatory action of progesterone on GnRH-I release from the hypothalamus (Guémené & Williams, 1986) and support for this proposal comes from the indirect evidence discussed in SECTIONS 3.3.4, 4.3.3 and 5.3. Thus injection of GnRH-I may induce a transitory positive feedback loop between LH and progesterone in laying hens, which does not induce a preovulatory-like surge of plasma LH in the absence of a fully mature preovulatory ovarian follicle.

The sexually differentiated profile of LH secretion

The profile of the plasma LH response to injection of GnRH-I in juvenile and adult chickens is partly determined by the biphasic pattern of LH secretion, comprising an initial spike followed by a plateau phase of LH release (SECTION 4.2.2.1; FIGURE 8.1). This biphasic response was well-defined *in vitro* (SECTION 4.2.2) but is not so clear *in vivo* (SECTION 3.2.2) because of the dynamics of metabolism and clearance from plasma of GnRH-I and LH *in vivo*. This pattern of LH secretion involves a GnRH-I-specific, and therefore a GnRH-receptor-dependent, mechanism because depolarising agents were unable to reproduce the biphasic pattern of secretion (SECTION 4.2.2.3). Evidence suggests that the decline of the plateau phase but not the spike phase of LH secretion from pituitary tissue from adult cockerels during a sustained stimulation with GnRH-I *in vitro* is due to depletion of secretory granules in the LH-gonadotroph cells (SECTION 4.3.3). However this biphasic pattern of LH secretion *in vitro* is not entirely representative of the profile of plasma LH *in vivo* due to the manner in which the pituitary gland is exposed to GnRH-I. Thus, the concentration of plasma GnRH-I to which the pituitary gland is exposed following intravenous injection of GnRH-I will decline exponentially, whereas a 20-minute exposure to sustained GnRH-I infusion is required to induce the plateau phase of LH secretion from the isolated pituitary gland. Such a circumstance may occur in laying hens during the preovulatory surge of LH but not in adult

cockerels or juvenile chickens, however not enough is known about the pattern of GnRH-I release in the chicken to confirm this. The sex difference in the GnRH-I-stimulated profile of plasma LH in adult chickens is therefore a function of the spike phase of LH secretion.

Sexual maturation in hens but not cockerels, is associated with a reduction in the size of the biphasic release of LH, and a marked change in the relative magnitude of the spike phase compared with the plateau phase of secretion. This suggests that 17ß-oestradiol acts in at least two ways to change the profile of LH release from pituitary glands of hens as they become sexually mature. Thus, there is a general suppression of both phases of LH secretion by 17ß-oestradiol through its action to decrease pituitary LHß-mRNA and the LH available for release (see SECTIONS 5.3 and 6.2.3), but this treatment also changes the spike phase-to-plateau phase ratio of LH secretion (CHAPTER 5). This proposal accounts for the difference in the spike-to-plateau phase ratio of LH released in response to GnRH-I between the LH profiles from pituitary glands of adult cockerels and laying hens (FIGURE 8.1), and between those of the juvenile and adult hen.

There are several ways in which 17ß-oestradiol could modify the intracellular signalling pathway to change the spike-to-plateau phase ratio of LH release from pituitary glands of adult cockerels. In the rat, protein kinase A (PKA) and protein kinase C (PKC) -dependent pathways participate in the second phase of LH secretion in response to GnRH, and these mechanisms are sexually differentiated (Chang *et al.*, 1987; Bourne, 1988; Das *et al.*, 1989; Fahmy *et al.*, 1989). Furthermore, this sexual differentiation is induced by 17ß-oestradiol (Bourne, 1988; Das *et al.*, 1989; Fahmy *et al.*, 1989). In the chicken, GnRH-I stimulates LH release from pituitary cell cultures from adults through a PKC-dependent pathway which was not sexually differentiated (SECTION 6.2.2). However the validity of this observation is uncertain because the cultures of adult pituitary cells were poorly responsive to GnRH-I. Only one previous study has examined the effect of 17ß-oestradiol on the intracellular signalling pathway by which GnRH-I induces LH secretion in the chicken. Treatment of pituitary cells from juvenile chickens with 17ß-oestradiol is reported not to affect the forskolin (activator of adenylyl cyclase) or TPA (activator of PKC) -induced release of LH (King *et al.*, 1989). This means that 17ß-oestradiol affects a signalling event(s) which occurs before the activation of adenylyl cyclase or PKC.

The endogenous activator of PKC, 1,2-diacylglycerol (DG) is formed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form DG and IP_3. Presumably, since the turnover of inositol polyphosphates in pituitary cells from juvenile chickens stimulated by GnRH-I is not affected by 17ß-oestradiol (King *et al.*, 1989), the turnover of DG would likewise be unaffected, which suggests that the phospholipase C-mediated hydrolysis of PIP_2 is not affected by 17ß-oestradiol. The event which precedes the activation of PKC and adenylyl cyclase is signal transduction by G-proteins in the cell membrane (SECTION 1.6.1). No studies have been made on the effects of gonadal steroids on G-protein function in the pituitary gland of the chicken, as they have in the rat (SECTION 1.6.4.3). The coupling of G-proteins with their enzymes or Ca^{2+} channels (Bouvier *et al.*, 1991; Mobbs *et al.*, 1991; Ravindra & Aronstam, 1992a) is influenced by

the fluidity of the membrane (SECTION 7.3). This relationship may be affected by 17ß-oestradiol in the chicken pituitary gland, to for example facilitate Ca^{2+} entry, and could explain how 17ß-oestradiol enhances the plateau phase of LH secretion from the pituitary gland of adult cockerels.

Another mechanism by which the sex difference in spike-to-plateau ratio of LH secretion is expressed, is through the relationship between the magnitude of the GnRH-I-stimulated spike phase of LH release, and the sexually differentiated ability to mobilise Ca^{2+} from intracellular stores of gonadotroph cells from adult but not juvenile chickens (CHAPTER 7). The ability of pituitary glands from adult cockerels and juvenile chickens of both sex, but not those from laying hens, to release LH in the absence of extracellular Ca^{2+} *in vitro*, suggests that this component of secretion diminishes during sexual maturation of the hen. Consequently, the increasing concentration of plasma 17ß-oestradiol in maturing hens could be important in the development of this sex difference in adult chickens. Studies using pituitary cells from rats show that the spike phase of LH secretion occurs almost exclusively through the GnRH-induced release of intracellular Ca^{2+}. The ability of pituitary glands from adult cockerels and juvenile chickens of both sex, but not those from laying hens, to release LH in the absence of extracellular Ca^{2+} *in vitro*, suggests that this component of secretion diminishes during sexual maturation of the hen. Consequently the increasing concentration of plasma 17ß-oestradiol in maturing hens could be important in the development of this sex difference in adult chickens (reviewed by Stojilkovic *et al.*, 1992b). However the GnRH-I-induced spike phase of LH release required three modes of Ca^{2+} flux in pituitary tissue of adult cockerels comprising of an intracellular Ca^{2+}-dependent component, and Ca^{2+} entry through L-type and non-L-type Ca^{2+} channels, whereas pituitary tissue from laying hens required only Ca^{2+} entry through non-L-type Ca^{2+} channels (CHAPTER 7).

Treatment with 17ß-oestradiol reduces the release of LH in response to GnRH-I from pituitary cells from juvenile chickens at a level of intracellular signalling between the formation of inositol polyphosphates and the mobilisation of the internal stores of Ca^{2+} (King *et al.*, 1989). This suggests that the inability of pituitary tissue from adult hens to secrete LH in Ca^{2+}-free medium could be due to a depressed release of intracellular Ca^{2+} (FIGURE 8.1). It is likely therefore that the profile of LH secretion from pituitary tissue of adult cockerels and laying hens result from a difference in their ability to mobilise the intracellular pool of Ca^{2+}.

These observations indicate a sex difference in the presence or absence of an intracellular store of Ca^{2+}, or a difference in the ability of second messengers to mobilise Ca^{2+} from these stores (FIGURE 8.1). The presence of internal pools of Ca^{2+} has been demonstrated in gonadotroph cells from juvenile chickens using thapsigargin which releases intracellular Ca^{2+} and stimulates LH secretion (Johnson & Tilly, 1991). This experiment has not been performed using pituitary cells from adult chickens because of the lack of a satisfactory method to culture functional gonadotroph cells. The second messengers which release intracellular Ca^{2+} are inositol 1,4,5-trisphosphate, and arachidonic acid and its metabolites (SECTION 1.6.1). A comparison of the GnRH-I-induced turnovers of these second messengers in the pituitary glands of adult cockerels and laying hens has

not been reported. However, the turnover of total inositol polyphosphates in pituitary cells from juvenile chickens is not affected by treatment with 17ß-oestradiol (King *et al.*, 1989). It is possible that the actions of these mediators of Ca^{2+} mobilisation are reduced due to a decrease in fluidity of the membranes of the internal stores. This could affect the availability of receptors for these second messengers on the endoplasmic reticulum-like structures (SECTION 7.3), through a decrease in sensitivity of IP_3-induced Ca^{2+} release which occurs by a reduced content of Ca^{2+} in the endoplasmic reticulum-like structure, or depends on IP_3-receptor heterogeneity through post-translational modification of the receptor (reviewed by Berridge, 1993). In any event, there is no evidence for mobilisation of intracellular Ca^{2+} in gonadotroph cells of the laying hen (FIGURE 8.1). It is concluded that sexual differentiation of the GnRH-I-induced initial phase of LH secretion *in vitro*, partly determines the profile of plasma LH in response to GnRH-I injection *in vivo*, and may be related to the sex difference in the ability of gonadotroph cells from adult chickens to mobilise the stores of intracellular Ca^{2+}. The resulting profile of plasma LH which follows is due to sexual differentiation of the mechanisms which extend the duration of increased plasma LH.

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